

POTENTIAL ROLE FOR HUMAN KCTD9 IN THE AUTOPHAGY
PATHWAY: A NOVEL AUTOPHAGOSOME-ASSOCIATED PROTEIN

by

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Abstract

KCTD9 belongs to the human potassium channel tetramerization domain (KCTD) family and is of unknown biological function. Our studies in yeast showed that the yeast homologue of KCTD, Whi2, is required to reduce TOR (target of rapamycin) kinase activity, thereby slowing down cell growth and inducing autophagy. Therefore, it was thought that KCTD9 may be associated to autophagosomes and playing a role in inducing autophagy.

For the first time, we were able to confirm that KCTD9's subcellular localization. KCTD9 forms cytoplasmic puncta that fall within the range of the size of autophagosomes. In order to identify these structures, KCTD9 was co-expressed with numerous cellular markers including endosomal and autophagy proteins. KCTD9 puncta did not co-localize with endosomal markers, but co-localized with LC3, a canonical marker of autophagosomes. Furthermore, KCTD9 co-localized with autophagy proteins involved in the nucleation and elongation of the autophagosome - Vps34, Beclin-1, Atg5, and Atg12. While KCTD9 puncta re-localize to LC3 marked structures under starvation conditions, it induces the formation of Vps34 and Atg5 puncta in complete media. Furthermore, KCTD9 associated with the endoplasmic reticulum (ER) suggesting it could be localizing to subdomains of the ER from which autophagosomes derive from.

Knockdown of KCTD9 leads to a decrease in the conversion of LC3-I to LC3-II indicating a stall in the autophagy pathway. KCTD9 could be playing a role in the initiation of this pathway by recruiting early autophagy proteins on the ER. Moreover, since it co-localized with E3-ligase Cullin-3 and acts a substrate adaptor, KCTD9 could be recruiting selective autophagic cargo.

These results suggest that KCTD9 is a novel autophagosome-associated protein playing a potential role in the induction of the autophagic pathway.

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Table of Contents

Title Page	i
Abstract	ii
Acknowledgments	iii
Table of Contents	iv
List of Tables	ix
List of Figures	x
Introduction	1
The KCTD Family	4
Cullin-3 Adaptors	5
Role in Disease	7
KCTD9	11
Autophagy	14
Regulation and Pathway	15
Innate and Adaptive Immunity	18
Viruses, Cancer, and Neurodegenerative Diseases	21
Methods	26
Results	29
Expression of KCTD9.....	29
KCTD9 self-interacts via BTB domain	30
KCTD9 co-localizes with Cullin-3.....	31
KCTD9 does not co-localize with endosomal markers.....	32

KCTD9 puncta localize to autophagosomes	32
KCTD9 localizes to subdomains of endoplasmic reticulum	35
Endogenous expression of KCTD9	36
KCTD9 knockdown leads to autophagy defect.....	37
Discussion	38
Figures	41
Figure 1	41
Figure 2	42
Figure 3.....	43
Figure 4.....	44
Figure 5.....	45
Table 1	46
Figure 6.....	47
Table 2	48
Figure 7.....	49
Figure 8.....	50
Figure 9.....	51
Figure 10.....	52
Figure 11.....	53
Figure 12.....	54
Figure 13.....	55
Figure 14.....	56
Figure 15.....	57

Figure 16.....	58
Figure 17.....	59
Figure 18.....	60
References.....	61
Curriculum Vitae.....	70

List of Tables

Table 1: Summary of co-localization results with endosomal markers46

Table 2: Summary of co-localization results with autophagy markers.....48

List of Figures

Figure 1: Outline of KCTD9 constructs	41
Figure 2: Expression of KCTD9 in COS7 cells leads to formation of cytoplasmic puncta	42
Figure 3: KCTD9 forms similar cytoplasmic structures in N2a cells	43
Figure 4: KCTD9 can self-interact via BTB mutant	44
Figure 5: KCTD9 co-localizes with Cullin-3	45
Figure 6: KCTD9 does not co-localize with endosomal markers	47
Figure 7: KCTD9 co-localizes with autophagosome marker LC3	49
Figure 8: Starvation induces LC3 puncta and increases co-localization with KCTD9	50
Figure 9: KCTD9 co-localizes with early autophagosome marker Vps34	51
Figure 10: KCTD9 induces Vps34 puncta in complete and starvation media.....	52
Figure 11: KCTD9 does not co-localize with FYVE-domain	53
Figure 12: KCTD9 co-localizes with autophagy protein Atg5	54
Figure 13: KCTD9 induces Atg5 puncta in complete and starvation media	55
Figure 14: KCTD9 full-length and mutant construct associate with endoplasmic reticulum marker Climp63	56

Figure 15: KCTD9 localizes to subdomains of the endoplasmic reticulum in COS7 cells	57
Figure 16: Endogenous expression of KCTD9 in N2a cells	58
Figure 17: KCTD9 siRNA knockdown leads to possible autophagy induction defect	59
Figure 18: Model of KCTD9's potential role in the autophagy pathway	60

Introduction

The human KCTD protein family is a group of proteins associated with numerous diseases of significant public health importance including cancer, neurodegeneration, and obesity (Liu et al., 2013). KCTD9 has been associated with breast cancer, metastasized prostate tumors, and autism (Naylor et al., 2005; Paris et al., 2006; Baron et al., 2006). More recently, KCTD9 has been found to promote liver inflammation in patients with chronic Hepatitis B Virus (HBV) (Chen et al., 2013). HBV is a major worldwide health concern because there are about 400 million individuals infected with the virus and 1 million deaths each year due to HBV (Grimm et al., 2013). HBV gets transmitted through exposure of infectious body fluids or blood and the virus replicates in the liver, but can eventually spread into the bloodstream (Yun-Fan Liaw et al., 2009). While acute illness includes liver inflammation, chronic infection can lead to liver cancer and cirrhosis (Yun-Fan Liaw et al., 2009). Immune-mediated liver damage plays a key role in chronic HBV infections and KCTD9 is thought to promote hepatic injury due to its possible role in NK cell activation (Grimm et al., 2013; Chen et al., 2013). Therefore, better understanding the biological function of KCTD9 will allow to clarify its potential as therapeutic target for patients with chronic HBV infections (Chen et al., 2013).

Our interest in the mammalian KCTD protein family stems from our lab's work done in yeast, where a poorly characterized general stress-response gene, yeast *WHI2* (whiskey-2, "wee-2"), is the top hit in our lab's genome-wide screen for regulators of nutrient-sensing and cell survival (Teng et al., 2013). Results show that *WHI2*-deficient (*Dwhi2*) yeast grow better on low amino acid media in comparison to wild type strains, but when exposed to stressful conditions, the yeast mutant has a tendency to undergo cell

death (Cheng et al., 2008; Teng et al., 2013). Furthermore, *WHI2* is required to reduce TOR (target of rapamycin) kinase activity specifically following amino acid withdrawal (Cheng et al., 2008; Teng et al., unpublished), thereby slowing down cell growth and inducing autophagy, a catabolic process involving the degradation of cell components through the lysosomal machinery (Klionsky et al., 2005). These findings are consistent with the possibility that the yeast Whi2 protein may be a new upstream negative regulator of TOR (Teng et al., unpublished). Alternatively, instead of being an upstream regulator, Whi2 could directly activate autophagy that feedback inhibits TOR in yeast (Mendl et al., 2011). Although the yeast Whi2 protein is reported to be fungi-specific, bioinformatics tools (HMM Pred search) identified the KCTD homologues in human genome (Teng et al., 2013).

So far very few studies on KCTD/Whi2 proteins have been undertaken to examine their specific functions. The KCTD/Whi2 proteins contain a BTB domain and therefore have been proposed to act as adaptors for the E3 ubiquitin ligase Cullin-3 (Pintard et al., 2005). BTB-domains are found in many proteins of diverse functions, but the best characterized BTB proteins are those in the cullin-RING ubiquitin ligases (CRLs) composed of a cullin, a RING protein (E3 activity) and a BTB/POZ adaptor such as Skp1 (Geyer et al., 2003). This E3 ubiquitin ligase complex plays an important role in polyubiquitination and degradation of certain protein substrates (Choo et al., 2012). Cullin-3 protein can bind an E3 ligase protein at its C-terminus and a BTB-containing protein at its N-terminus (Stogios et al., 2005). In turn, the BTB protein is thought to recognize specific targets for ubiquitination/neddylolation (Pintard et al., 2003). Recently, Cullin-3 was reported to play a role in regulating the late steps of the endo-lysosomal

trafficking and to be important for a late step in influenza virus uncoating (Huotari et al., 2012). Interestingly, mammalian and yeast TOR localize to lysosomal/vacuolar membranes (Gong et al., 2011), and vesicle trafficking pathways are known to be required for nutrient responses that regulate TOR in yeast, though the details remain unresolved (Dechant et al., 2008). Based on these findings, combined with our lab's yeast Whi2 studies, my project was to investigate the potential role of human KCTD9 in responding to nutrients and the autophagy pathway. Our hypothesis is to determine where along the autophagy pathway KCTD9 participates, to examine its subcellular localization and ultimately to determine whether it is an essential autophagy protein.

The KCTD Family

The human potassium channel tetramerization domain (KCTD) protein family consists of 26 members, mostly with unknown biological functions (Liu et al., 2013). KCTD proteins are cytoplasmic proteins that seem not binding to K⁺ or functioning as potassium channels, but received this name since their N-terminal BTB/POZ domain shares the greatest sequence homology with the BTB-like tetramerization domain (T1 domain) of voltage-dependent potassium channels (Liu et al., 2013; Dementieva et al., 2009). While the KCTD family members have a conserved Bric-a-brack, Tram-track, Broad complex (BTB) domain, their C-termini can be quite variable in sequence and length. The BTB domain was first identified in *Drosophila melanogaster* and has protein-protein interaction motif for homodimerization or heterodimerization (Dementieva et al., 2009). There are about 400 BTB domain-containing proteins in the human genome and 9 in yeast (Perez-Torrado et al. 2006), in which the BTB is usually located at the N-terminus that may contain additional domains, such as zinc-finger domains, as kelch repeats, and FYVE (Fab1, YOTB, Vac1, and EEA1) domains (Stogios et al., 2005). The BTB domain allows for the KCTD proteins to self-assemble and to bind to other proteins (Dementieva, 2009). For example, the X-ray crystal structure of KCTD5 shows that it can assemble into five subunits through its BTB domain (Dementieva, 2009). As more research is conducted on this BTB domain-containing family, results show the involvement of KCTDs in cytoskeleton regulation, transcriptional repression, interaction with E3-ligase complex Cullin-3, and human disease (Liu et al., 2013).

Cullin-3 Adaptors

Due to their BTB domains, KCTD proteins could possibly act as substrate adaptors for the Cullin-3 ubiquitin ligase and aid in the ubiquitination of substrate proteins (Furukawa et al., 2005). There are eight cullin proteins that act as molecular scaffolds in the assembly of a multi-subunit Cullin-RING E3 ubiquitin ligase (CRL) to ubiquitinate cellular proteins (Sarikas et al., 2011). There are three enzymatic steps involved in ubiquitinating a protein - the activation occurs by the ubiquitin-activating enzyme E1, which allows for the transfer of the activated ubiquitin to the ubiquitin-conjugating enzyme E2 (Bosu et al., 2008). Then the ubiquitin ligase E3, which binds the substrate, can bring the substrate and E2 close enough for the E2 to conjugate the ubiquitin onto the substrate (Bosu et al., 2008).

The cullin protein helps to connect the substrate, through the adaptor protein, and the RING finger portion of the CRL, allowing for facilitation of the transfer of ubiquitin to the substrate by the RING-bound E2 enzyme (Hochstrasse et al., 1996). The cullin proteins play a crucial role in this ubiquitination process therefore regulating numerous cellular processes including signal transduction, transcriptional control, cell growth, and tumor suppression (Sarikas et al., 2011).

Cullin-3 has been reported to control many biological functions in mammalian cells such as, cell death, protein trafficking, transcriptional activation, and stress responses (Genschik et al., 2013). In addition, cullin-3 has been associated with human metabolic disease, dystrophies, pseudohypoaldosteronism and cancer (Genschik et al., 2013). The role of cullin-3 in protein trafficking is of key interest to our research as we consider the possible involvement of KCTD proteins in the autophagy pathway. Recent

studies by Huotari et al. (2012) show that cullin-3 is involved in the late steps of the endosomal pathway by regulating the transport of late endosomes to the lysosome. To study the function of cullin-3 in endosomal trafficking, influenza A virus (IAV) and epidermal growth factor receptor (EGFR) are tracked from their internalization from the cell surface (Huotari et al., 2012). When cells are depleted of cullin-3 using siRNA, both IAV and the EGFR are able to be internalized into late endosome/lysosome compartments marked by Lamp1 (Huotari et al., 2012). However, the degradation of EGFR and uncoating of the virus is delayed and cells begin to accumulate abnormal looking late endosomes and became highly vacuolized with Rab7-marked endosomes (Huotari et al., 2012). These results suggest that cullin-3 is involved in endosome maturation and it is thought that ubiquitination is involved in the process (Huotari et al., 2012).

A handful of KCTD proteins have been identified as candidate substrate adaptors for cullin-3 including KCTD11, KCTD21, KCTD6, KCTD5, and KCTD7 (Liu et al., 2013). The Hedgehog signaling pathway is known to be deregulated in tumors including medulloblastoma (Di Marcotullio et al., 2004). Gli1 and Gli2 are proteins involved in the Hedgehog signaling pathway and their genes are deacetylated by HDAC (histone deacetylase) to upregulate the Hedgehog pathway (Canettieri et al., 2010). KCTD11 was reported to act as an adaptor for HDAC1 to be degraded by the cullin-3 ubiquitin complex leading to suppression of the Hedgehog activity (Canettieri et al., 2010). KCTD11 has been associated with medulloblastoma when it undergoes allelic deletion or is expressed at low level (Di Marcotullio et al., 2004). KCTD 21 and KCTD6 have also been found to act as substrate adaptors for the histone deacetylase protein 1 in the

Hedgehog signaling pathway (De Smaele et al., 2010). While KCTD5 has also been shown to bind cullin-3, the nature of the substrates recognized by KCTD5 for possible targeting for ubiquitination is not known (Bayon et al., 2008). Similarly, KCTD7 can be co-immunoprecipitated with cullin-3, but no target substrate has been identified (Azizieh et al., 2011). It is suggested that KCTD7 may increase the conductance of voltage-gated potassium channels, possibly by degrading through cullin-3 ubiquitination by the proteasome (Azizieh et al., 2011), but this mechanism remains unproven. Further research is needed to verify if KCTD family proteins function dependently to cullin ligases, and investigate which substrates are targeted by the KCTD proteins, which would give more insight into the functions of the KCTD family (Liu et al., 2013).

Role in Disease

The KCTD family members have been associated with several diseases such as medulloblastoma, breast carcinoma, neurological diseases, obesity, and pulmonary inflammation (Liu et al., 2013). This section will specifically discuss the role of KCTD11, KCTD7, and KCTD13 in human diseases. KCTD11, located on chromosome 17p13.2 has been identified as a tumor suppressor and this portion of the chromosome is commonly deleted in human medulloblastoma, a malignant pediatric brain tumor (Smaele et al., 2004). These tumors are thought to arise from neuronal progenitor cells during the development of the cerebellum (Wechsler-Reya et al., 2001). The Hedgehog signaling pathway, which is responsible for regulating the differentiation of the progenitor cells during development of the cerebellum, is not turned off in medulloblastoma, leading to uncontrolled growth of the progenitor cells leading to malignancy (Argenti et al., 2005).

As a cullin-3 substrate adaptor, KCTD11 is suggested to inhibit the Hedgehog pathway by preventing the nuclear localization of the Gli proteins to prevent activation of Hedgehog target genes (Canettieri et al., 2010). In vitro experiments showed that overexpression of KCTD11 can inhibit medulloblastoma formation in cell lines and colony formation in soft agar (Di Marcotullio et al., 2004). Furthermore, KCTD11 can prevent xenograft tumor growth in vivo (Di Marcotullio et al., 2004). Therefore, KCTD11 is considered to be a tumor suppressor due to its ability to inhibit the Hedgehog signaling pathway and prevent its deregulation that leads to medulloblastoma (Di Marcotullio et al., 2004).

KCTD7 mutations define a subset of patients with progressive myoclonus epilepsy (EPM), EPM3, first identified by Bogaert et al. (2007) in a Moroccan family with this autosomal recessive disorder. Patients with EPM3 usually present with seizures between the age of 16 to 24 months and overtime experience neurological weakening leading to an early death (Krabichler et al., 2012). Bogaert and colleagues, found that the Moroccan family members had a mutation in exon 2 of KCTD7 leading to a change from an arginine codon to a stop codon (R99X) revealing that KCTD7 is the potential genetic cause of EPM3 (Bogaert et al., 2007). Since this study, KCTD7 mutations have been identified in 24 patients, each with homozygous or compound heterozygous mutations including missense mutations R94W (Krabichler et al., 2012), R94T, D115Y, N273I, W289X (Kousi et al., 2012) and L108M (Lemke et al., 2012). Related disorders reported to be associated with KCTD7 mutations include acute onset of myoclonus, ataxia, and neuronal ceroidlipofuscinosis (Blumkin et al., 2012; Staropoli et al., 2012). While the exact function of KCTD7 is still unknown, expression of KCTD7 decreases the

excitability of murine cortical neurons, but also kills cells (Azizieh et al., 2011). Overexpressed GFP-KCTD7 punctae localize in the cytoplasm and to the plasma membrane. However, when the mutant form of KCTD7 (Δ R184C) was overexpressed, in neurons KCTD7 formed cytoplasmic aggregates, but did not localize to the plasma membrane (Staropoli et al., 2012). This mutant form of KCTD7 disrupts the ubiquitin-proteasome system (UPS) leading to the accumulation of protein aggregates (Staropoli et al., 2012). These data are consistent with the possibility that KCTD7 may play a role in protein trafficking and when its mutated form contributes to changes in plasma membrane composition and accumulation of cytosolic proteins leading to neuronal dysfunction and death (Staropoli et al., 2012).

Genetic disorders have been linked to copy number variants (CNV) and the region of the 16p11.2 chromosome has been found to make individuals more susceptible to neurological defects when deleted or duplicated (Stankiewicz et al., 2010). This region of chromosome 16 has 29 genes, including KCTD13, and duplication or deletion of this region has been associated with schizophrenia, epilepsy, autism spectrum disorder (ASD) (Weiss et al., 2008). When human KCTD13 was overexpressed in zebrafish embryos it resulted in a microcephaly phenotype, which is associated with 16p11.2 duplication events (Golzio et al., 2012). Microcephaly occurs when there is less proliferation of neuronal progenitor cells, but an increase in apoptosis while the brain is developing leading to smaller head size (Golzio et al., 2012). On the other hand, suppressing the expression of KCTD13 in the zebrafish embryos caused macrocephaly, large head size, due to an increase in progenitor proliferation and no change in apoptosis, which is associated with 16p11.2 deletion events (Golzio et al., 2012). These results indicated that

KCTD13 plays a major role in head size phenotypes, which are associated with 16p11.2 CNV, by regulating the initial steps of neurogenesis (Golzio et al., 2012). In humans, KCTD13 is associated with autism, some cases of schizophrenia and childhood obesity (McCarthy et al., 2009; Walters et al., 2010). Researchers found a family with ASD and other neurological disorders that had a 118-kb deletion in 16p11.2, which included the MVP, CDIPT1, SEZ6L2, ASPHD1 and KCTD13 genes (Crepel et al., 2010). This deletion can actually be inherited from an asymptomatic parent or occur spontaneously. involving multiple loci including KCTD13 (Golzio et al., 2012). It is possible that as the polymerase delta-interacting protein 1 (PDIP1), KCTD13 has the ability to interact with proliferating cell nuclear antigen and through this interaction has the ability to regulate the cell cycle during neurogenesis (He et al., 2001). More research will allow for better understanding of why deregulation of KCTD13 leads to neurological abnormalities (Golzio et al., 2012).

KCTD9

KCTD9 is considered to be a medium-sized KCTD family member made up of 389 amino acids, and like other KCTD proteins it has a BTB near the N-terminus (amino acids 91-181) (Skoblov et al., 2013). Additionally, KCTD9 has a KHA domain in its N-terminus (amino acids 1-64) and four sets of eight overlapping pentapeptide repeats (amino acids 118-377) (UniProt, 2014; Skoblov et al., 2013). The KHA domain is normally found in plant potassium channels and is needed for potassium channel interaction (Zimmermann et al., 2001). While both prokaryotes and eukaryotes have pentapeptide repeats, eukaryotes usually only contain one gene encoding pentapeptide repeats typically containing eight copies of the sequence similar to A(D/N)LXX (Skoblov et al., 2013). The most well studied pentapeptide repeat proteins are mycobacterial MfpA and enterococcal homologs of Qnr (Hedge et al., 2011; Merens et al., 2009). Three-dimensional structures of these pentapeptide repeats reveal that they mimic the size, charge, and shape of DNA, allowing the bacteria to be resistant to quinolones, an antibacterial drug, but binding up DNA-binding proteins (Merens et al., 2009). In addition, bacterial pentapeptide repeat proteins have the ability to interact with DNA gyrase, an enzyme that relieves strain when DNA is being unwound by a helicase, and prevent it from relieving DNA strain (Hedge et al., 2011; Merens et al., 2009). It has been suggested that the pentapeptide repeats in KCTD9 also can act as a DNA mimic (Skoblov et al., 2013). KCTD9 has been reported to interact with MED20, which is part of the mediator complex that acts as a scaffold for assembly of the RNA polymerase II transcriptional complex (Merens et al., 2009). Furthermore, genomic profiling studies have reported reduced or no expression of KCTD9 mRNA in breast and metastasized

prostate tumors (Naylor et al., 2005; Paris et al., 2006). Microarray studies have found declining KCTD9 mRNA expression during the progression of childhood autism (Baron et al., 2006). However, the actual molecular function of KCTD9, or any other KCTD family member, is still unknown.

Recently, Chen et al. (2013) studied the expression of KCTD9 in blood and liver samples from patients with either mild chronic hepatitis B (CHB) or HBV-induced acute-on-chronic liver failure (HBV-ACLF). HBV-ACLF was seen in numerous patients with chronic hepatitis B infection who have developed liver failure due to hepatic necrosis (Chen et al., 2013). It is thought that immune-mediated mechanisms are involved in the necrosis process especially cytotoxic T cells and natural killer (NK) cells (Chen et al., 2013). Peripheral blood mononuclear cells (PBMCs) were examined and KCTD9 is found to be mainly localized in the cytoplasm in mild CHB patients, but has both cytoplasmic and nuclear localization in HBV-ACLF patients (Chen et al., 2013). It is thought that KCTD9 translocates to the nucleus in HBV-ACLF patients, where it is suggested to be a contributing factor in the severity of the disease (Chen et al., 2013). Interestingly, other KCTD proteins, including KCTD1, 10, and 15 have been reported to translocate to the nucleus and interact with transcription factors, however, this has not been established for KCTD9 (Ding et al., 2009; Liu et al., 2009; Zarelli et al., 2013).

KCTD9 is highly expressed in peripheral and hepatic NK cells of HBV-ACLF patients in comparison to those with mild CHB and the high level of KCTD9 expression in patients with chronic HBV was linked to the severity of liver damage (Chen et al., 2013). PBMC gene expression profiling showed that 263 genes from 8600 were highly upregulated in HBV-ACLF patients and KCTD9 was one of these genes (Chen et al.,

2013). Such high expression of KCTD9 may cause alterations in NK cell function and thereby increasing hepatic damage (Chen et al., 2013). Transfecting NK92 cells with KCTD9 caused a significant increase in NK cell cytotoxic activity and release of IFN- γ (Chen et al., 2013). However, when shRNA against KCTD9 was transfected in NK92 cells, there was a decrease in NK cell activation and cytotoxicity of hepatic cells (Chen et al., 2013). These results suggest the potential role of KCTD9 in the activation of NK cells, but the mechanism is still unclear (Chen et al., 2013). However, KCTD9 serves as a potential therapeutic target for HBV-ACLF patients (Chen et al., 2013). Another study further consolidated the role of KCTD9 in hepatic damage in HBV-ACLF while testing an anti-hepatic failure compound (AHFC) using the mouse model of acute hepatic failure infected with MHV-3 virus (Huang et al., 2009). AHFC decreased the expression of KCTD9 thereby preventing hepatic damage (Huang et al., 2009). Other immune-related genes like *KCTD9* that were decreased in their expression in response to AHFC include Bcl-2, IL-8, IL-6, and IFN- γ (Huang et al., 2009).

Bioinformatic (STRING 8.2) analysis predicts that KCTD9 may interact with SHB (SH2 domain-containing adaptor protein B), which is involved in signal transduction pathways of T cell receptors, IL-2, and apoptosis signaling (Lindholm et al., 2002). It is plausible that KCTD9 acts as a substrate adaptor protein, especially since it has been reported that the KCTD proteins could be substrate adaptors for the E3-ligase Cullin-3 (Geyer et al., 2003).

Autophagy

Autophagy is a regulated cellular process that involves the bulk degradation of cytoplasmic components including long-lived proteins and whole organelles (Mizushima, 2007). This process is highly conserved from yeast to mammals and is different from the endocytic pathway, which involves the lysosomal degradation of extracellular and plasma membrane proteins (Mizushima, 2007). There are three types of autophagy, which include macroautophagy, microautophagy, and chaperone-mediated autophagy (Ravikumar et al., 2010). Microautophagy and chaperone-mediated autophagy both directly take cytoplasmic components to the lysosome, but macroautophagy (hereafter referred to as autophagy) involves the formation a double-membraned structure called the autophagosome (Ravikumar et al., 2010). While autophagy occurs in the cell at a basal level, it can be upregulated in response to stress situations such as starvation, pathogens, and endoplasmic reticulum (ER) stress (Ravikumar et al., 2010). The formation of the autophagosome allows for the elimination of cytoplasmic materials including harmful protein aggregates and microbes and recycling of nutrients (Ravikumar et al., 2010).

While the membrane source for the formation of autophagosomes is still debated, the ER is generally agreed to be one of the membrane sources (Geng et al., 2010). Autophagy is disrupted in yeast strains that are defective in the early stages of the secretory pathway involving ER-to-Golgi transport, indicating a role for the ER as a membrane source (Geng et al., 2010). Pre-autophagosome membrane projections that grow from the ER are surrounded by rough ER (Codogno et al., 2012). When cells have a block in the maturation of pre-autophagosomal structures there is an accumulation of ER-associated early autophagic structures (Codogno et al., 2012). Aside from the ER,

mitochondria have also been suggested to be another membrane source (Codogno et al., 2012). Research in mammalian cells has shown that the outer mitochondrial membrane can be contiguous with autophagosomes, and fluorescently labeled mitochondrial lipids appear to be transferred to the autophagosome (Codogno et al., 2012). In addition, recently it has been found that there are connections between the ER and mitochondria for the transfer of proteins between the two structures (Tooze et al., 2010). These newly characterized ER-mitochondrial connections could also act as a source of membrane for autophagosomes (Tooze et al., 2010). Other possible sources of membrane include the plasma membrane, the Golgi complex and endocytic vesicles (Codogno et al., 2012; Geng et al., 2010).

The Autophagy Pathway

The mTOR (mammalian/metabolic target of rapamycin) pathway is a key regulator of mammalian autophagy (Ravikumar et al., 2010). The mTOR signaling molecule is a Ser/Thr kinase that actively suppresses autophagy in rich nutrients, and stimulates the initiation of mRNA translation, ribosome biogenesis, transcription, and cytoskeletal reorganization (Ravikumar et al., 2010). The mTOR protein is found in two main complexes – mTORC1, which responds to nutrient status and negatively regulates autophagy, and mTORC2, which is less well characterized but a role in autophagy is not clear (Ravikumar et al., 2010). mTORC1 inhibits autophagy in the presence growth factors, energy, and amino acids, but when there is a lack of amino acids, growth factors, or an increase in the AMP/ATP ratio, mTORC1 is inhibited, allowing for the initiation of autophagy (He et al., 2009). In nutrient rich conditions mTORC1 phosphorylates Unc-51-

like kinase 1 (ULK1) and 2 (ULK2), and autophagy-related protein 13 (Atg13) thereby inhibiting autophagy induction (He et al., 2009). During starvation conditions, mTORC1 is inhibited and ULK1 and ULK2 undergo autophosphorylation and phosphorylate Atg13 and focal adhesion kinase family-interacting protein of 200 kD (FIP200) (He et al., 2009). This phosphorylation step is thought to be crucial to cause the translocation of ULK1/2, Atg13, and FIP200 complex to pre-autophagosomal structures (Alers et al., 2012). The ULK1/2, Atg13, and FIP200 complex is the most upstream of the autophagy pathway known and is crucial for the recruitment of the next autophagy-related protein complexes (Alers et al., 2012).

After the induction of autophagy, nucleation and formation of the phagophore membrane, a double-membrane structure that eventually develops into an autophagosome, is mediated by the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, which includes the vacuolar protein sorting 34 protein (Vps34/PI3K), p150, Atg14, and Beclin 1 (He et al., 2009; Tooze et al., 2010). Vps34 is responsible for producing phosphatidylinositol 3-phosphate (PI3P) within subdomains of the ER, a membrane source for autophagosomes, which become nucleation sites for autophagosome biogenesis (Devereaux, 2013). Furthermore, these PI3P sites are important in recruiting to the isolation membrane other autophagy proteins including those with FYVE domains (which bind PI3P), PX domains, and WD-repeat domains (Devereaux, 2013). These proteins such as, WD repeat domain phosphoinositide-interacting (WIPI) protein, act as scaffold proteins for the recruitment of additional autophagy proteins required for elongation and closure of the autophagosome (Devereaux, 2013). Beclin 1, another PI3K complex member, is regulated by B-cell

lymphoma/leukemia (Bcl-2), an antiapoptotic protein (He et al., 2009). In nutrient-rich conditions, Bcl-2 sequesters Beclin 1, but Beclin 1 dissociates from Bcl-2 for autophagy induction (He et al., 2009). The Vps34/PI3K complex facilitates the recruitment of two ubiquitin-like conjugation systems to the phagophore – Atg12-Atg5-Atg16 and LC3 (He et al., 2009).

The first ubiquitin-like conjugation system involved in the elongation and closure of the phagophore is the Atg12-Atg5-Atg16 complex (Geng et al., 2008). Atg7 activates the ubiquitin-like protein Atg12 and then Atg10, conjugates Atg12 to Atg5 (Geng et al., 2008). The Atg12-Atg5 conjugate binds to Atg16, a coiled-coil protein, and this complex forms a tetramer attaching to the phagophore (Mizushima et al., 2003). The second ubiquitin-like system is the LC3 conjugation system, where Atg4, a cysteine protease, cleaves LC3 at the C-terminus to form the cytosolic LC3-I with a C-terminal glycine residue (Fujita et al., 2008). LC3-I gets conjugated to phosphatidylethanolamine (PE) by Atg7 and Atg3, creating the lipidated form LC3-II (Mizushima, 2007). LC3-II gets attached to both the inner and outer faces of the phagophore membrane at sites determined by the Atg12-Atg5-Atg16 complex (Kabeya et al., 2000). LC3 has the ability to control the size of the autophagosome by determining the membrane curvature (Xie et al., 2008). Furthermore, LC3 is considered to be the gold-standard marker for the detection of autophagosomes by immunofluorescence microscopy, and the conversion of LC3-I to LC3-II is widely used to monitor autophagy induction on immunoblots (Rosenfeldt, 2012).

Once the autophagosome formation is complete, Atg4 cleaves LC3 from its PE moiety on the outer membrane of the autophagosome, recycling the LC3 back into the

cytoplasm (Kirisako et al., 2000). Other autophagy proteins, such as Atg12 and Atg5, also get cleaved off the autophagosome, but the mechanism of how they are recycled is still not clear (Kirisako et al., 2000). The final step of the process involves the fusion of the autophagosome to the lysosome (Jager et al., 2004). This fusion is mediated by the small GTPase Rab7 and LAMP-2, a lysosomal membrane protein, but the mechanism has not been fully characterized (Jager et al., 2004). After the autophagosome-lysosome fusion occurs, a number of lysosomal/vacuolar acid hydrolases degrade the contents of the autophagosome (Jager et al., 2004). Certain molecules, such as amino acids, get recycled back to the cytoplasm for protein synthesis and other cellular functions during starvation (He et al., 2009).

Innate and Adaptive Immunity

While autophagy plays a role in numerous cellular functions, its role in inflammation and the immune response is starting to gain more attention (Kuballa et al., 2012). In addition, since KCTD9 plays a potential role in NK cell activation (Chen et al., 2013) and possibly the autophagy pathway, it is appropriate to highlight the role autophagy in the innate and adaptive immune systems. More research has begun to focus on the role of autophagy in immune function due to the association of autophagy genes and inflammatory disorders such as Crohn's disease (Kuballa et al., 2012). It has been shown that autophagy plays a crucial role in intracellular pathogen sensing and defects in autophagy can render an individual more susceptible to infection (Shin et al., 2010). Furthermore, autophagy functions by bridging the innate and adaptive immune systems since it plays a role in thymic selection, antigen presentation, lymphocyte homeostasis

and survival, and cytokine production (Kuballa et al., 2012).

In general, MHC class II antigens are produced through endocytosis of the protein antigens from extracellular regions and undergo lysosomal degradation before loaded onto the MHC class II molecule (Rammensee et al., 1997; Neefjes, 1999). More recently it has been shown that cytoplasmic proteins and pathogens can end up into the lysosome by autophagy and be presented by MHC class II molecules (Paludan et al., 2005). MHC class II molecules from human B cells are found to be intracellular molecules and under starvation conditions, which induces autophagy; more of these peptides are presented (Dengjel et al., 2005). In addition, the loading compartment of the MHC class II molecules in thymic epithelial cells and dendritic cells co-localize with autophagosomes, further supplementing the idea that autophagy plays a role in antigen presentation (Kasai et al., 2009). Furthermore, studies have shown that dendritic cells require autophagy in order to process and present extracellular pathogens onto MHC class II molecules (Lee et al., 2010). Dendritic cells are responsible for presenting pathogen antigens in order to prime T cells (Lee et al., 2010). However, mice with Atg5-deficient dendritic cells are unable to prime their CD4⁺ T cells after been infected with Herpes Simplex Virus (Lee et al., 2010). The Atg5-deficient dendritic cells are unable to properly process and present antigens on their MHC class II molecules, cathepsins transport to MHC class II processing compartments is hindered, and there is a delay in fusion between autophagosomes and lysosomes (Lee et al., 2010).

Numerous autophagy factors play essential roles in B and T cell functions (Kuballa et al., 2012). When Atg7 is deleted in the hematopoietic cells of mice, they have a reduced number of B and T lymphocytes in the periphery indicating the role of Atg7 in

lymphocyte maintenance (Mortensen et al., 2010). Specific studies in B cells have shown the need for Atg5 in B cell development and maintenance of the B-1a B cell lineage (Miller et al., 2008). Mice with B-cell specific deletion of Atg5 have a much lower number of B-1a B cells in the periphery in compared to other B cell lineages (Miller et al., 2008). Furthermore, mice with Atg5-deficient B cell progenitors are not able to develop into mature B cells because they cannot transition from pro- to pre-B cells resulting in cell death (Miller et al., 2008). Another autophagy protein, Beclin 1, is known to play a role in early B cell development (Arsov et al., 2011). Beclin 1-deficient mice have fewer lymphoid progenitor cells including common lymphoid progenitors and hematopoietic stem cells in the bone marrow compared to wild-type mice (Arsov et al., 2011). However, Beclin 1-deficient mice still have a normal level of peripheral B cells (Arsov et al., 2011). Therefore, while Beclin 1 and Atg5 plays an important role in B cell development, only Atg5 and Atg7 are involved in the maintenance of peripheral B cells (Kuballa et al., 2012).

Similarly, autophagy proteins including Beclin 1, Atg3, Atg5, and Atg7 play a critical role in the development, maintenance, and survival of T cells (Kuballa et al., 2012). Mice that have Atg3-, Atg5-, or Atg7-deficient T cells have T cells with less thymic cellularity (Jia et al., 2011). In addition, naïve peripheral CD4⁺ and CD8⁺ T cells that lack Atg3, Atg5, or Atg7 have reduced peripheral numbers, undergo increased apoptosis, and have difficulty proliferating (Jia et al., 2011; Hubbard et al., 2010). When these autophagy-deficient T cells were examined, it was found that they had supranumerary mitochondria and increased ROS levels (Hubbard et al., 2010; Pua et al., 2009). When T cells mature and go to peripheral organs they clear their mitochondria,

unless autophagy is impaired (Pua et al., 2009). Mitophagy, a form of autophagy that removes damaged mitochondria plays an important role in clearing proliferating T cells' mitochondria when they shift to glycolytic metabolism after being activated (Mao et al., 2011). As more research is performed to unveil the role of autophagy in innate and adaptive immune pathways it is becoming clear that autophagy proteins play a crucial role in the immune system. Furthermore, understanding the role the KCTD family plays in the autophagy pathway could lead to the discovery of possible immune-related function the KCTD proteins.

Viruses, Cancer, and Neurodegenerative Diseases

Defective autophagy has been implicated in an increasing number of human diseases, from viral infections to cancer and neurodegeneration (Levine et al., 2008). Autophagy plays anti-viral and pro-viral roles in the life cycle of numerous viruses (Kudchodkar et al., 2009). Anti-viral roles include the targeting of viral proteins by autophagy proteins for lysosomal degradation (Kudchodkar et al., 2009). Autophagy proteins also initiate innate and adaptive immune responses against viral pathogens (Kudchodkar et al., 2009). On the other hand, certain viruses use the autophagy pathway in order for intracellular growth or non-lytic cellular egress (Kudchodkar et al., 2009). More recently, research emphasis is given to interfere with the beneficial aspects of autophagy for viruses destruction (Kudchokar et al, 2009). Many viruses have developed ways to escape their targeted degradation by autophagy (Kudchokar et al, 2009). Herpes simplex virus type 1 (HSV-1) encodes a protein called ICP34.5 that can interact with the autophagy protein Beclin 1 in order to prevent autophagy induction (Talloczy et al.,

2006). Certain RNA viruses including poliovirus, dengue virus-2 (DENV2), rotavirus, and influenza virus A prevent autophagosome-lysosome fusion (Schmid et al., 2007). The influenza virus A viral M2 protein inhibits autophagosome-lysosome fusion and thereby can prevent MHC class presentation of viral particles (Schmid et al., 2007). A handful of DNA viruses including adenovirus, Epstein-Barr virus (EBV), and hepatitis B virus (HBV) actually activate the autophagy pathway in order to increase their viral replication (Jiang et al., 2011). Due to KCTD9's association with HBV understanding the relationship of HBV and autophagy could help provide more insight into the function of KCTD9.

It is known that HBV enhances autophagy to promote the replication of its proteins. HBV is able to use both direct and indirect triggers in order to induce autophagy (Li et al., 2011). Direct mechanisms involved viral elements that trigger autophagy (Li et al., 2011). Indirect mechanisms involve the cell inducing autophagy due to cellular stress caused by the virus (Li et al., 2011). Researchers have shown that HBV expression is linked to autophagy induction and specifically the formation of early autophagosomes (Sir et al., 2010). However, even though autophagy is induced, autophagic protein degradation does not increase (Sir et al., 2010). However, even though autophagy is induced, autophagic protein degradation does not increase (Sir et al., 2010). Therefore it is thought that HBV acts at the early stages of autophagy and possibly delays autophagosome maturation (Sir et al., 2010). Further research will allow for the better understanding of how exactly HBV induces the early stages of autophagy and this may provide more insight into the possible role being played by KCTD9 in chronic HBV infections.

Due to the homeostatic functions of autophagy, disruption of the process can allow for the promotion of tumorigenesis (Matthew et al., 2009). Since autophagy removes damaged organelles and proteins and can limit cell growth and genomic instability, it functions as a tumor suppression mechanism (Matthew et al., 2009). Beclin 1 heterozygous mice were tumor-prone meaning that it is a haplosufficient tumor suppressor gene (Qu et al., 2003). When Beclin 1 is overexpressed thereby over stimulating autophagy tumor development can be inhibited (Liang et al. 1999). When autophagy is defective, there is an accumulation of p62/SQSTM 1 protein aggregates, damaged mitochondria, and misfolded proteins leading to the creation of reactive oxygen species (ROS) (Matthew et al., 2009). This can cause DNA damage, which leads to genomic instability and the development of tumors (Matthew et al., 2009). On the other hand, cancer cells use autophagy to develop stress tolerance and survive (Semenza et al., 2010). Cancer cells can induce autophagy in order to cope with metabolic and cytotoxic stressors such as hypoxia and nutrient deprivation (Semenza et al., 2010). Autophagy-deficient cancer cells have difficulty surviving when exposed to metabolic stress in comparison to autophagy-proficient cells (Matthew et al., 2009). Human pancreatic cancer cell lines and tumor specimens have increased levels of autophagy, which allow for tumor growth by sustaining energy production. Inhibiting autophagy in these cells actually caused tumor regression and allowed for longer survival of genetic mouse models (Yang et al., 2011). Autophagy can be considered as a key target for cancer treatment due to its role in tumor suppression and in some instances tumor progression (Granville et al., 2007). By stimulating autophagy using rapamycin, a drug that inhibits mTOR, 90% reduction in lung tumors have been observed in mice (Granville et al., 2007)

while inhibiting autophagy in tumor cells has allowed for anticancer drugs to work more efficiently (Zhineng et al., 2011).

In neurons, it appears that autophagy plays an even more crucial role in the maintenance of cellular homeostasis since neurons are postmitotic and cannot get rid of abnormal protein accumulation through cell division (Son et al., 2012). Most neurodegenerative diseases are characterized by misfolded protein aggregates including Alzheimer's disease, Parkinson's disease, tauopathies, Huntington's disease, spinocerebellar ataxias (Ross et al., 2004). Mice with autophagy defects in neurons start to develop intracellular inclusions in the brain and neurodegenerative symptoms even though they did not express any disease-causing aggregate-prone proteins (Congcong et al., 2006). *Atg5^{-/-}* and *Atg7^{-/-}* deficient mice are born normally, but start to develop aging-related neurodegeneration including tremors, limb claspings, and ataxic walking (Congcong et al., 2006). In addition, these mice lost their Purkinje cells and ubiquitin-positive inclusion bodies in the cerebral cortex, cerebellum hypothalamus, which are signs of neurodegeneration (Congcong et al., 2006). Furthermore, these autophagy-deficient mice die within 28 weeks (Congcong et al., 2006). Autophagy is considered to be a potential target to prevent the accumulation of certain protein aggregates responsible for neurodegeneration (Ravikumar et al., 2004). Rapamycin has been used as a potential therapeutic agent for clearance of aggregate proteins and reduction of neurodegenerative symptoms in both *Drosophila melanogaster* and mice (Ravikumar et al., 2004). However, long-term use of rapamycin has been reported to cause adverse side effects, such as immunosuppression, and poor wound healing (Winslow et al., 2007). Further insight into the role autophagy plays in neurodegeneration and other possible therapeutic

agents will allow the development of more adequate treatment options for patients suffering with neurodegenerative disease (Winslow et al., 2007).

Our goal is to better understand the autophagy pathway and its role in human diseases. While the KCTD protein family still remains largely understudied, they have been shown to be associated with a number of human diseases. Determining whether the KCTDs play a role in the autophagy pathway could provide more insight about the function of these proteins and the autophagy pathway, and KCTDs could serve as possible therapeutic targets.

Methods

Plasmid Constructs

Full-length human KCTD9 cDNA was PCR amplified and inserted into expression vectors pDB59 and pHML8 using In-Fusion cloning. Mutant constructs were created by developing primers in order to PCR amplify either the N-terminus region or C-terminus region of KCTD9 cDNA and inserted into expression vector pDB59 using In-Fusion cloning. All four constructs were verified by sequence analysis. RFP-Rab5 and myc-ULK1 plasmids were purchased from Clontech. GFP-Rab7, LAMP1-RFP, RFP-LC3, mCherry-Atg5, Vps34-FLAG, and FYVE-dsRed were obtained as gifts from other laboratories.

Cell Culture and Autophagy Induction

African Green monkey kidney cells (COS7) and mouse neuroblastoma (N2a or Neuro-2) cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and 1% penstrep. Cultures were incubated at 37°C, 5% CO₂. Autophagy was induced under starvation conditions by incubating cultures with amino acid-free and serum-free DMEM for 4 hours or by treating with rapamycin (50nM) for 2 hours.

Transfection and Immunostaining

Cells were plated on coverslips in a 24-well plate 24 hours before transfection. Cells were transfected with a total of 0.375ug of DNA, which was incubated with 0.75ul of Lipofectamine 2000 (Invitrogen) and 50ul of Opti-MEM medium per well. The mix was

directly added to 500ul of Opti-MEM in each well and incubated for 5 hours after which the Opti-MEM was removed and DMEM was added. After 19 hours cells were washed with cold PBS and were fixed using 4% paraformaldehyde for 10-15 minutes at room temperature. Cells were washed again with cold PBS and permeabilized with 0.2% Triton-X100 for 5 minutes at room temperature. Cells were blocked for 30 minutes with 2% goat serum at 4°C and then treated with primary antibody (1:1000 dilution) for 2 hours at room temperature. Cells were washed with cold PBS and were blocked once again with goat serum for 30 minutes at 4°C before treating with secondary antibody (1:1000 dilution) for 1 hour. Cells were washed with cold PBS and were treated with DAPI (1:4000 dilution) for 10 minutes at room temperature and then mounted onto slides using Gold Anti-fade Reagent. Slides were left at 4°C overnight before acquiring images.

Fluorescence Microscopy and Data Analysis

A Nikon 90i fluorescence microscope was used to acquire images at 100x objective and analyzed using Volocity analysis software (PerkinElmer). Three independent experiments were quantified using the 60x objective and are presented as mean \pm SD of the mean for 300-400 cells per sample.

Protein Co-immunoprecipitation

Two DNA plasmids were transfected into COS7 cells on a 6 well plate in equal amounts. 24 hours later, cells were lysed using cold isotonic lysis buffer (0.2% NP-40, 142.5 mM KCl, 5mM MgCl₂, 10 mM HEPES, 1 mM EGTA at pH 7.5) with protease inhibitor cocktail and phosphatase inhibitor. Cell lysates were incubated at 4°C with GFP antibody

(Invitrogen) overnight. The next day Protein-G-agarose beads (Santa Cruz) were incubated for 4 hours at 4°C. Cell lysates were washed four times with cold PBS, suspended in 1x Sample buffer (50 mM Tris-HCl pH 6.6, 2% SDS, 10% glycerol, 1% β -mercaptoethanol, 0.02 % bromophenol blue) and vortexed 3 times. Lysates were boiled for 2 minutes at 100°C before performing western blot analysis.

Lysate collection and Western blot Analysis

Mouse lysates were obtained from 5-week-old NIH Swiss mouse and suspended in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris pH 8.0). N2a or COS7 cells were transfected with DNA plasmids and cell lysates were collected 24 hours later and suspended in 1x Sample buffer. Once lysates were collected they were vortexed 5 times and boiled for 3 minutes at 100°C. Lysates were loaded onto 12% SDS-PAGE gel, transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories), and detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare Life Sciences).

RNA interference of KCTD9

N2a cells were seeded in 12-well plate for 19 hours before transfection. A negative control scramble siRNA or KCTD9 Smartpool siRNA (Dharmacon) was transfected using Dharmafect according to the manufacturer's protocol. After 72-96 hours of transfection, cell lysates were collected using 1x Sample buffer, vortexed 3 times, boiled for 5 minutes at 100°C and subjected to western blot analysis.

Results

Expression of human KCTD9 forms cytoplasmic puncta in COS7 and N2a cells

In order to characterize human KCTD9 two full-length constructs were made: one with a N-terminal HA-tag and another with a N-terminal GFP-tag (Fig. 1). These constructs were expressed in COS7 cells and resulted in the formation of cytoplasmic puncta. Both HA-KCTD9 and GFP-KCTD9 predominantly formed “vesicle-like” puncta, which had an average diameter of 1.25 μm (Fig. 2A (right), 2C). Autophagosomes have been reported to range from 0.5-1.5 μm (Mizushima et al., 2002). In addition, expression of constructs resulted in the formation of smaller puncta, with an average diameter of 0.7 μm , and more elongated structures as shown for the HA-KCTD9 construct (Fig. 2A (left and middle)). HA-KCTD9-containing structures were quantified as small puncta, elongated puncta, and “vesicle-like” puncta (Fig. 2B). To confirm these structures are not artifact, an empty GFP expressing vector was transfected into COS7 cells and resulted in a diffuse cytoplasmic (Fig. 2C). Therefore, expression of HA-KCTD9 and GFP-KCTD9 in COS7 cells form cytoplasmic puncta remaining to be identified.

In order to confirm these structures, HA-KCTD9 was transfected into N2a cells resulting in the formation of similar cytoplasmic structures - round puncta, elongated puncta, and “vesicle-like” puncta (Fig. 3A). When quantified at 19 hours post-transfection, HA-KCTD9 mainly formed the small round puncta. However, 40 hours post-transfection there was an increase in the number of cells with “vesicle-like” puncta (Fig. 3B). Once again these structures were similar in size to autophagosome - the round puncta had an average diameter of 0.63 μm and the “vesicle-like” puncta were 1.46 μm . In comparison to 19 hours, there was more HA-KCTD9 protein expressed at 40 hours

(Fig. 3C). Therefore, it can be concluded that human KCTD9 localizes to the cytoplasm forming small puncta that can be transformed into larger “vesicle-like” structures.

KCTD9 self-interacts via BTB domain

To further characterize KCTD9, two mutant constructs were created : a N-terminal HA-tagged BTB domain-containing construct and a N-terminal HA-tagged pentapeptide repeat containing construct (Fig. 1). The BTB domain containing construct included the first half of the protein, from amino acids 1 to 194 (hereafter referred to as KCTD9-194x). While the pentapeptide repeat containing construct included the C-terminal of KCTD9 from amino acids 186 to 389 (hereafter referred to as KCTD9 Δ N). When KCTD9-194x was transfected in COS7 (Fig. 4A left) or N2a (Fig. 4B left) cells it formed very small puncta. These puncta seem to be lined up in an organized fashion as though they are vesicles being trafficked, but live imaging will be needed to test this hypothesis. On the other hand, KCTD9 Δ N in both COS7 (Fig. 4A right) or N2a (Fig. 4B right) cells form a diffuse yet string-like network in the cytoplasm. This structure is very reminiscent of endoplasmic reticulum, which has been reported to look like an interconnected network of tubules (Voeltz et al., 2002). These mutant constructs will be used to determine the nature of the cellular structures containing KCTD9 and to study BTB domain protein interaction.

KCTD9 contains a BTB domain, a protein-protein interaction domain that has been reported to allow for numerous cellular functions such as targeting proteins for ubiquitination, transcriptional regulations, and cytoskeleton dynamics. BTB domains can

allow for proteins to either self-associate or interact with non-BTB proteins (Stogios et al., 2005). In order to confirm that KCTD9 can self-associate through its BTB domain, a co-immunoprecipitation was performed where full length GFP-KCTD9 was used to examine if it can pull down HA-KCTD9-194x, HA-KCTD9 Δ N or HA-KCTD13-141x. Full length GFP-KCTD9 was able to interact with its own BTB domain since it pulled down the KCTD9-194x mutant (Fig. 4C left). As expected, it did not pull down the KCTD9 Δ N mutant lacking the BTB domain (Fig. 4C middle). Interestingly, full length GFP-KCTD9 was not able to pull down the BTB containing mutant KCTD13-141x (Fig. 4C right). These results confirm that KCTD9 can self-associate through its BTB domain and furthermore this interaction is specific for its own BTB domain since it did not interact with KCTD13's BTB domain. In addition, these results suggest that KCTD9 can make larger "vesicle-like" structures by interacting with its BTB domain.

KCTD9 co-localizes with Cullin-3

The KCTDs have been reported to be substrate adaptors for the E3 ubiquitin ligase cullin-3 via their BTB domain (Furukawa et al., 2005). Cullin-3-myc was expressed in COS7 cells and it formed puncta that were reminiscent of HA-KCTD9-194x, but the puncta seemed slightly smaller (Fig. 5A). When Cullin-3-myc was co-expressed with HA-KCTD9 they co-localized in large "vesicle-like" structures (Fig. 5B). The HA-KCTD9 structures alone form smaller vesicles, but co-expression with Cullin-3-myc induced the formation of larger structures. It is possible that these larger structures could contain proteins for which KCTD9 is a substrate adaptor. Furthermore, HA-KCTD9-194x puncta co-localized with Cullin-3-myc since this was the BTB-containing

portion of KCTD9 (Fig. 5C). These results suggest that KCTD9 and Cullin-3 have the ability to localize to the same structures and may possibly interact. Co-immunoprecipitation experiments will confirm potential interaction between KCTD9 and Cullin-3.

KCTD9 does not co-localize with endosomal markers

Huotari et al., reported the role of Cullin-3 in endosomal maturation and BTB domain containing-proteins possibly in endolysosomal and recycling pathways (2012). Therefore, HA-KCTD9 and HA-KCTD9-194x constructs were co-expressed with known endosomal markers in N2a cells. Rab5 localizes to early endosomes and the cytoplasmic side of the plasma membrane (Gorvel et al., 1999). When Rab5-RFP was co-expressed with HA-KCTD9 or HA-KCTD9-194x they formed distinct puncta revealing no co-localization (Fig. 6A, 6B). Furthermore, the Rab5-RFP puncta localized to a distinct part of the cytoplasm away from the HA-KCTD9 puncta (Fig. 6A). In addition, HA-KCTD9 did not co-localize with GFP-Rab7 (Fig. 6C) nor with Lamp1-RFP (Fig. 6D), which are markers of late endosomes and lysosomes (Jager et al., 2004). Treatment of CCCP to induce mitophagy or starvation to induce macroautophagy did not induce co-localization of KCTD9 structures with endosomal markers (data not shown). KCTD9 may then not play a direct role in the endocytic pathway.

KCTD9 puncta localize to autophagosomes

Whi2, the yeast homologue of KCTD9, is thought to play a role in nutrient sensing and possibly in the induction of autophagy (Teng et al., unpublished).

Furthermore, Cullin-3 has been reported to be a LC3 interacting protein (Behrends et al., 2010). Therefore, in order to test our hypothesis that KCTD9 is involved in autophagy, KCTD9 was co-expressed with autophagy markers in COS7 cells (Table 2). LC3 is considered to be the most widely accepted marker of autophagosomes and can form small puncta in the cytoplasm at basal conditions and puncta can increase when autophagy is induced (Levine et al., 2011; Kabeya et al., 2000). In complete media, RFP-LC3 resulted in the formation of about 9-13 puncta per cell in three individual experiments (Fig. 7A). When autophagy was induced by amino acid and serum withdrawal (starvation) for 4 hours the number of RFP-LC3-labelled autophagosomes increased to about 17-21 puncta per cell in three individual experiments (Fig. 7A). HA-KCTD9 puncta co-localized with RFP-LC3 in complete media and under starvation conditions there was an increase in the amount of co-localization (Fig. 7B, 7C, 8A). Although not all of the HA-KCTD9 puncta localized to the autophagosomes, it is possible that a certain amount of KCTD9 translocated to autophagosomes, more when autophagy was induced.

In order to see if KCTD9 co-localizes with other autophagy markers, different markers of the autophagy process were tested. Vps34-FLAG, an early marker of the autophagy process, was expressed in COS7 cells and formed an extended network in cells grown in complete media, but under starvation conditions puncta were observed (Fig. 9A). When co-expressed with HA-KCTD9 in complete media, KCTD9 induced the formation of puncta positive for Vps34 (Fig. 9B). When starved, there was more co-localization seen between KCTD9 and Vps34 (9C, 10A). These results suggest that KCTD9 may be playing a role in autophagy induction by virtue of its co-localization with the PI3K Vps34. Furthermore, HA-KCTD9-194x induces the change in structure of

Vps34-FLAG, which is normally diffuse but co-expressed causes it to form small puncta like HA-KCTD9-194x (Fig. 9D). HA-KCTD9-194x did not cause a morphological change with other autophagy proteins including LC3 and Atg5 (data not shown). Interestingly, KCTD9 does not co-localize with the FYVE-domain, a domain found in autophagy proteins such as WIPI-1. FYVE-domain containing proteins can bind to PI(3)P, therefore acting downstream of the PI3K Vps34 (Kutateladze et al., 2001). Expressing FYVE-dsRed in COS7 cells results in the formation of small cytoplasmic puncta and under starvation conditions results in the formation of more cytoplasmic puncta (Fig. 11A). These cytoplasmic puncta are very reminiscent of the puncta formed by KCTD9. However, when co-expressed with KCTD9 in complete media or starvation, the puncta do not co-localize, but instead seem to be next to each other (Fig 11B, 11C). Furthermore, KCTD9 puncta seem to cluster into larger structure as they are outcompeted by FYVE. It is possible that the FYVE-domain and KCTD9 are competing for similar regions of PI(3)P and that both are involved in the early formation of autophagosomes.

Another autophagy protein that KCTD9 co-localized with was Atg5, which is required for the lipidation of LC3 with phosphatidylethanolamine (PE) and recruitment of LC3 to autophagosomes (Hanada et al., 2007). When expressed in COS7 cells mCherry-Atg5 is diffuse, but under starvation conditions starts to form puncta (Fig. 12A). Following co-expression of mCherry-Atg5 and KCTD9, HA-KCTD9 induced the formation of mCherry-Atg5 puncta that are also positive for KCTD9 in complete media, and more co-localization in puncta containing these 2 proteins was visible under starvation conditions (Fig. 12B, 12C, 13A). These results suggest that KCTD9, which

localized with LC3-marked autophagosomes, can possibly induce autophagosome formation and co-localizes with autophagosome proteins Vps34 and Atg5.

KCTD9 localizes to subdomains of the endoplasmic reticulum

The ER is considered to be a possible membrane source for the autophagosome. Since KCTD9 co-localized with early autophagosomal marker Vps34, it is possible that KCTD9 localizes to sites of autophagosomal formation on the ER (Geng et al., 2010). In order to visualize the ER, Climp63-FLAG, a cytoskeleton-linking ER membrane protein, was expressed in COS7 cells forming an interconnected network of ER (Fig. 14A) (Nikonov et al., 2007). When Climp63-FLAG was co-expressed with HA-KCTD9, the “vesicle-like” puncta localized within the interconnected network of Climp63-FLAG (Fig. 14B). Furthermore, when Climp63-FLAG was co-expressed with HA-KCTD9 Δ N, which was thought to be reminiscent of ER, HA-KCTD9 Δ N structure followed a similar pattern to that of Climp63-FLAG (Fig. 14C).

To further investigate whether KCTD9 associates with the ER, a flipped-Reticulon4b construct was used. Reticulon4b is an ER associated protein that plays a role in the ER’s tubular morphology (Voeltz et al., 2005). The flipped-Reticulon4b construct causes the collapse of the ER around the nucleus. When the HA-flipped-Reticulon4b construct was expressed in COS7 cells, the ER condensed around the nucleus as marked by the dotted lines (Fig. 15A). Co-expression of HA-flipped-Reticulon4b and GFP-KCTD9 not only resulted in GFP-KCTD9 structures localizing within the condensed ER, but “vesicle-like” puncta were no longer present (Fig. 15B). By changing the shape of the ER, KCTD9 structures were also changed indicating that KCTD9 possibly is associated

to certain subdomains of the ER. On the other hand, expression of a dsRed construct containing the FYVE domain localize to the outer region of the HA-flipped-Reticulon4b (Fig. 15C). The dsRed-FYVE makes puncta that seem to localize at the edge, but the puncta of FYVE are not distorted like that of KCTD9. These results suggest that KCTD9 may localize to different regions of the ER in comparison to FYVE domain-containing proteins.

Endogenous expression of KCTD9 in N2a cells

While expression of KCTD9 in two different cell lines have provided us some insight into the localization of the protein, and possible function of KCTD9 in the autophagy pathway, remains be demonstrated. One autophagy assay involves looking at LC3-II levels, as a sign of active autophagy (Klionsky et al., 2012). In order to perform the autophagy assay, we used a commercial antibody (Genetex Cat. # GTX119656) against KCTD9 that cross-reacts with endogenously expressed KCTD9. Three cell lines were tested and N2a cells had a high level of endogenous KCTD9 expression (Fig. 16A). In addition, a five-week old NIH Swiss mouse was dissected to obtain tissue lysates (Fig. 16A), showing endogenous expression of KCTD9 in the cerebellum, liver, and spleen lysates.

In order to confirm endogenous expression of KCTD9 in N2a cells, an immunofluorescence experiment was performed using the commercial antibody. Endogenous KCTD9 puncta look similar to overexpressed KCTD9 in N2a cells (Fig. 16B left). However, certain puncta had a nuclear localization (Fig. 16B right). Cheng et al. reported that KCTD9 was mainly localized in the cytoplasm of PBMCs, but could be

translocated to the nucleus in HBV-ACLF patients (2013). Therefore, it is possible that KCTD9 has the ability to translocate into the nucleus as illustrated by endogenous staining, but overexpression may end up preventing the translocation as only cytoplasmic puncta were visible.

KCTD9 knockdown leads to autophagy defect

When autophagy proteins are knocked down, there is a change in LC3 levels - detectable by western blot. When KCTD9 levels were decreased in N2a cells there was a concomitant decrease in LC3-II levels, in comparison to the untransfected control and scramble siRNA. In the untransfected control and scramble siRNA cells, bands for LC3-I and LC3-II were apparent suggestive of basal level of autophagy occurring in the cells. However, when KCTD9 is partially knocked down, there is a decrease in the amount of LC3-II (Fig. 17). While these are preliminary results, it is possible that there is a decrease in the conversion of LC3-I to LC3-II due to a block in the pathway. KCTD9 may be playing a role in the early induction stages of the pathway, and in the absence of this protein, there is a decrease in activities of autophagy.

Discussion

This research is the first to specifically focus on KCTD9's morphology, subcellular localization, and function. KCTD9 forms distinct cytoplasmic puncta in COS7 and N2a cells, which fall within the range of autophagosomes. Furthermore, KCTD9 puncta range in size from small round to puncta to more vesicle-like structures that may possibly be carrying cargo. As a Cullin-3 substrate adaptor, KCTD9 has the ability to bind certain substrates and target them for degradation. While Cullin-3 ubiquitinated proteins usually are degraded by through proteasomal machinery, it is possible that Cullin-3 plays a role in trafficking pathways (Huotari et al., 2012). With its newly discovered role in endosomal trafficking, Cullin-3 along with KCTD9 may be involved in the autophagy pathway by either targeting certain proteins for degradation or acting as chaperones for autophagy-related proteins. In the future, co-immunoprecipitation experiments can be performed in order to confirm the interaction between Cullin-3 and KCTD9. Then determining if Cullin-3 co-localizes with autophagy markers and if deletion of Cullin-3 results in autophagy defects.

In order to determine KCTD9's subcellular localization, it was co-expressed with numerous cellular markers. While KCTD9 did not localize with endosomal markers, it localized with autophagy markers. In numerous cells, KCTD9 and LC3, an autophagosome-marker, co-localized and stimulation of autophagy resulted in an increase of co-localization. Furthermore, KCTD9 co-localized even more strongly with other autophagy proteins like Vps34 and Atg5. These results indicate that KCTD9 may be an autophagosome-associated protein playing a potential role in the early stages of the autophagy pathways.

Some autophagosomes are known to be derived from the ER and KCTD9 associated with ER marker like Climp63 and remains associated with the ER upon shape change of the ER induced by Flipped-Reticulon 4b. These results suggest that KCTD9 may localize to certain subdomains of the ER where autophagosomes derive from (Fig. 18). In order to confirm these results it will be interesting to determine how KCTD9 associates with the ER and its specific role in the derivation of autophagosomes. Our hypothesis is that KCTD9 interacts to certain subdomains of the ER through its C-terminus because of the similar ER morphology. An autophagy assay was used in order to determine if KCTD9 plays a functional in the autophagy pathway, as suggested by its localization to autophagosomes. Partial knockdown of KCTD9 results in a reduction in LC3-II levels suggesting for a functional role of KCTD9 in the early induction of the autophagy pathway. Due to the co-localization with nucleation and elongations autophagy markers with KCTD9 and its effect in the autophagy assay we believe that KCTD9 may play a potential in the induction of the autophagy pathway. While more research needs to be done in order to confirm the role of KCTD9 in the autophagy pathway these results suggest that KCTD9 may be a novel autophagosome-associated protein.

This research will pave the way for further studies on the KCTD9's role in autophagy and its link it to immunology and virology. KCTD9 has been implicated in NK cell activation, but the exact mechanism is unknown (Chen et al., 2013). It is possible that NK cell activation occurs through autophagy involving KCTD9. Autophagy-associated proteins such as Atg5 have been found to be involved in B and T cell activation (Kuballa et al., 2012). However, more research needs to be done in order to

understand the role of autophagy in NK cell activation and proliferation. Furthermore, KCTD9 expression increases in patients with chronic HBV infections, and HBV is known to induce autophagy for viral replication to occur (Chen et al., 2013; Li et al., 2011). If KCTD9 is involved in the induction of autophagy, then it may be targeted by HBV to stimulate viral replication. A lot more attention needs to be dedicated to specify the exact function of KCTD9 because it could act a therapeutic target for autophagy-related disease and chronic HBV infections.

A



B

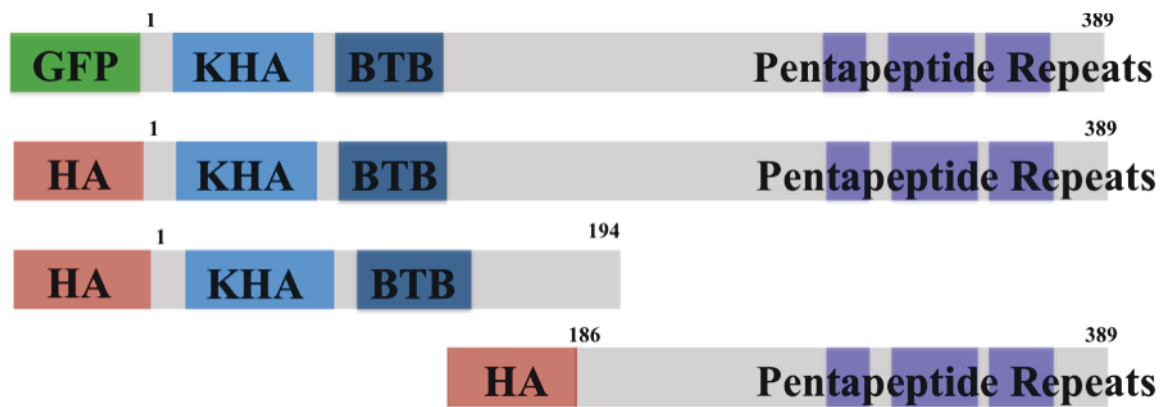


Figure 1. KCTD9 constructs design. (A) KCTD9 full-length protein of 389 amino acids with domains - KHA, BTB, and pentapeptide repeats. (B) Tagged versions of KCTD9 full-length protein and two mutant proteins - HA-KCTD9-194x and HA-KCTD9ΔN.

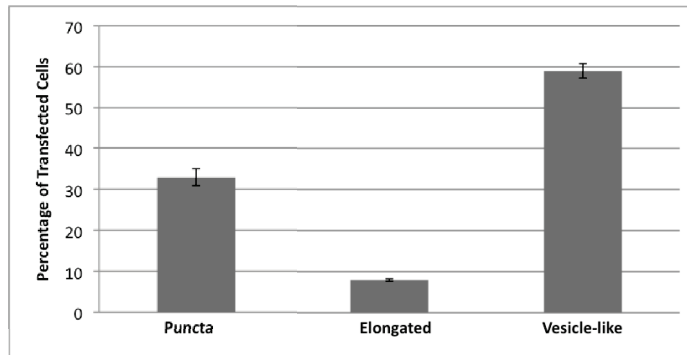
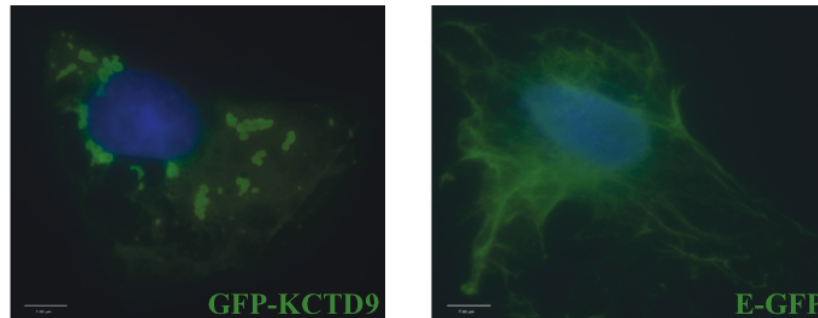
A**B****C**

Figure 2. Expression of KCTD9 in COS7 cells leads to formation of cytoplasmic puncta. (A) Immunofluorescence microscopy of COS7 cells transfected with HA-KCTD9 (anti-HA, red) for 19 h; nuclei stained with DAPI (blue). (B) Quantification of various HA-KCTD9 puncta found in COS7 cells 19 h post-transfection (n=3). (C) Immunofluorescence microscopy of COS7 cells transfected with GFP-KCTD9 (anti-GFP, green) or E-GFP (anti-GFP, green) for 19 h; nuclei stained with DAPI (blue).

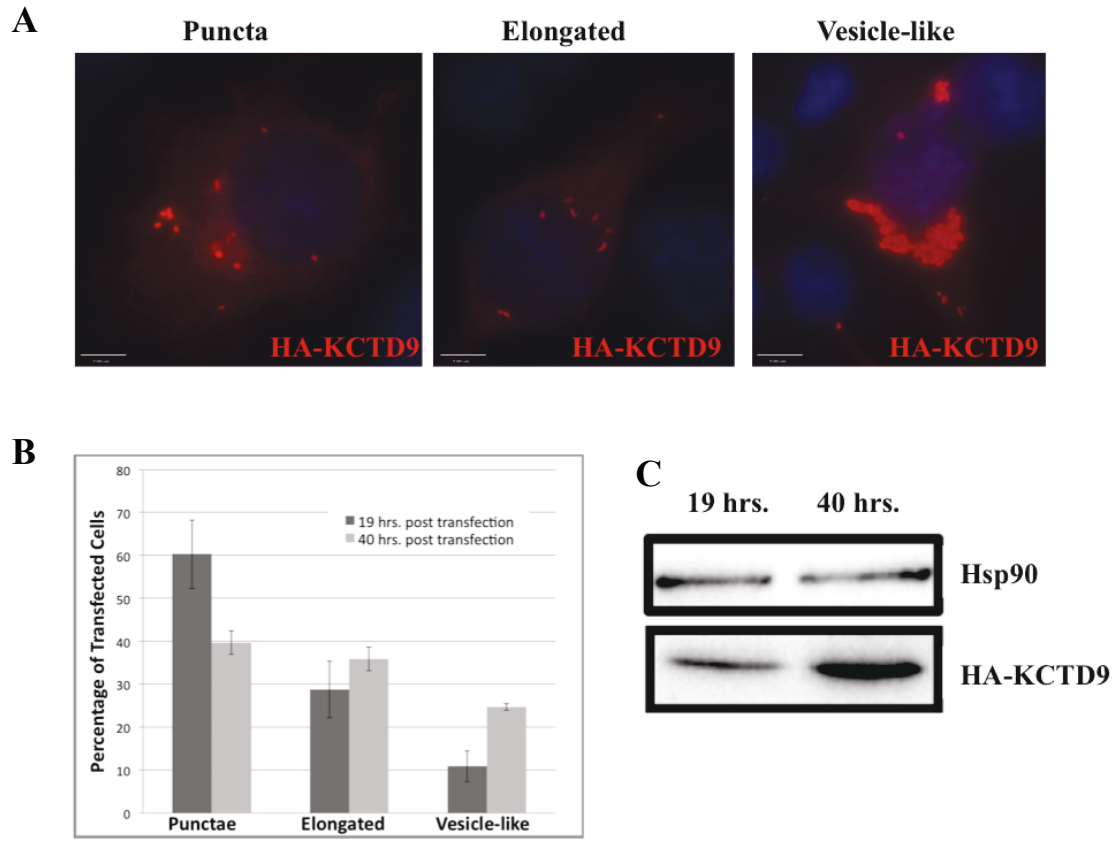


Figure 3. KCTD9 forms similar cytoplasmic structures in N2a cells.

(A) Immunofluorescence microscopy of N2a cells transfected with HA-KCTD9 (anti-HA, red) for 19 h; nuclei stained with DAPI (blue). (B) Quantification of various HA-KCTD9 puncta found in N2a cells 19 h or 40 h post-transfection (n=3). (C) Western blot showing level of KCTD9 (anti-HA) at 19 or 40 h post-transfection; Hsp90 detected with anti-Hsp90.

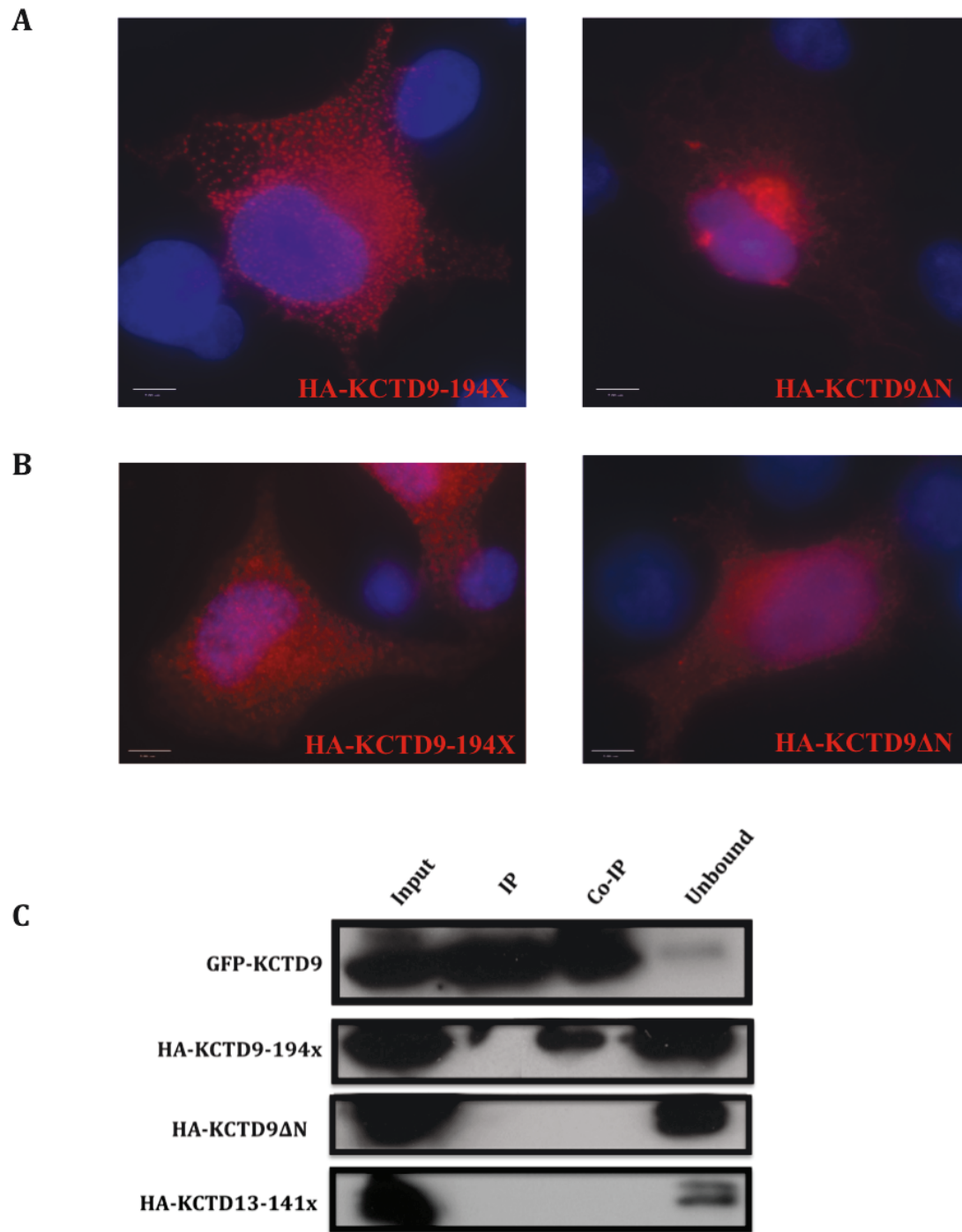


Figure 4. KCTD9 can self-interact via BTB mutant. (A) KCTD9 mutants in COS7 cells detected with anti-HA antibody (red); nuclei stained with DAPI (blue). (B) KCTD9 mutants in N2a cells detected with anti-HA antibody; nuclei stained with DAPI (C) Co-immunoprecipitation of GFP-KCTD9 and BTB-containing mutant HA-KCTD9-194x.

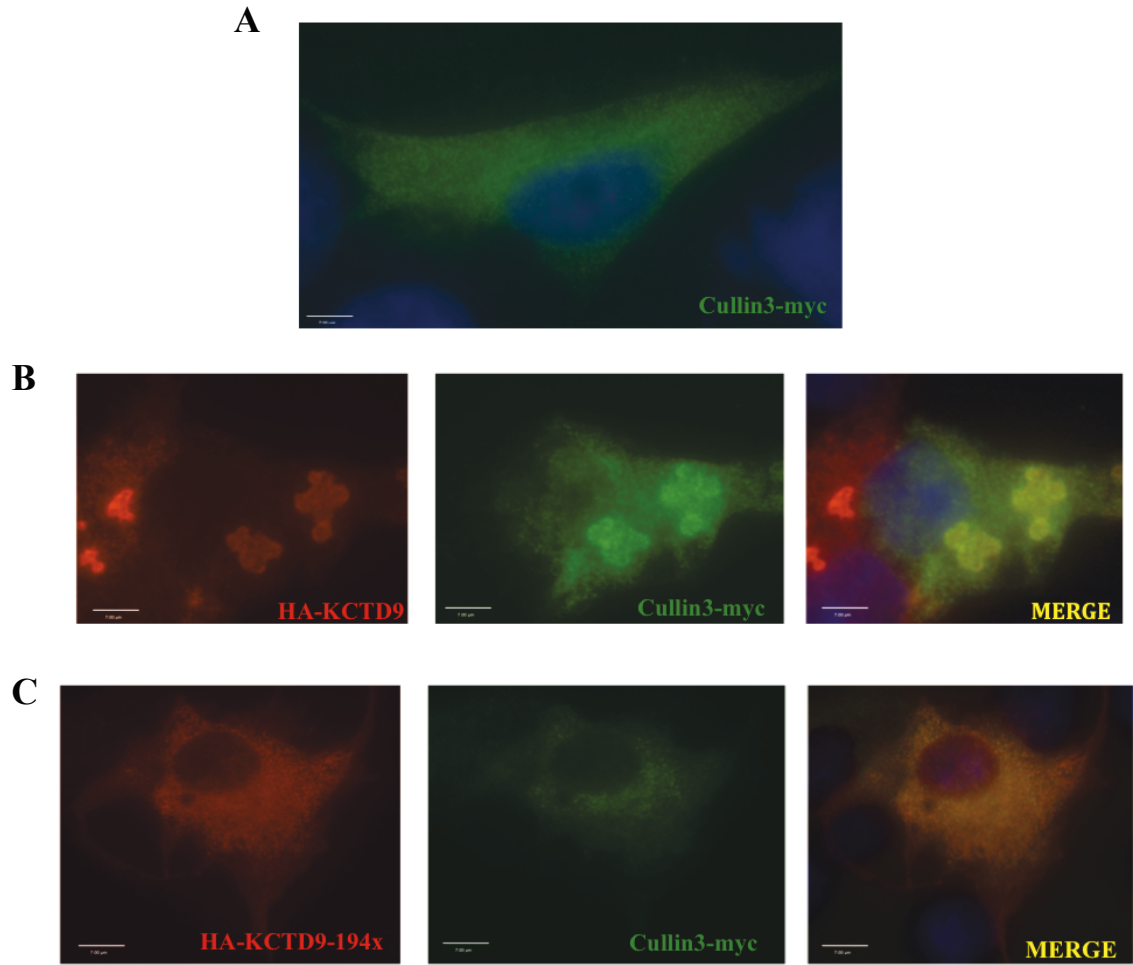


Figure 5. KCTD9 co-localizes with Cullin-3. (A) Immunofluorescence microscopy of COS7 cells transfected with Cullin-3-myc (anti-myc, green); nuclei stained with DAPI (blue). (B) Co-expression of HA-KCTD9 (anti-HA, red) and Cullin-3-myc (anti-myc, green) 19h post-transfection; nuclei stained with DAPI (blue) (C) Co-expression of KCTD9 mutant (anti-HA, red) and Cullin3-myc (anti-myc, green) post 19 h; nuclei detected with DAPI (blue).

Early Endosomal Marker		
Condition	No treatment	Treatments (Chloroquine or Starvation Media)
RFP-Rab5	No co-localization	No co-localization
MVB/Late Endosomal Markers		
GFP-Rab7	No co-localization	No co-localization
GFP-Rab11A	No co-localization	No co-localization
GFP-Rab11B	No co-localization	No co-localization
Cullin3-myc	Co-localization	-
Lysosomal Marker		
Lamp1-RFP	No co-localization	No co-localization

Table 1. Summary of co-localization results between endosomal markers and KCTD9. Numerous immunofluorescence experiments were performed in order to determine if endosomal markers and KCTD9 co-localize as outlined above. These experiments were performed in COS7 and N2a cells with no treatment or treatment - chloroquine or an autophagy stimulus.

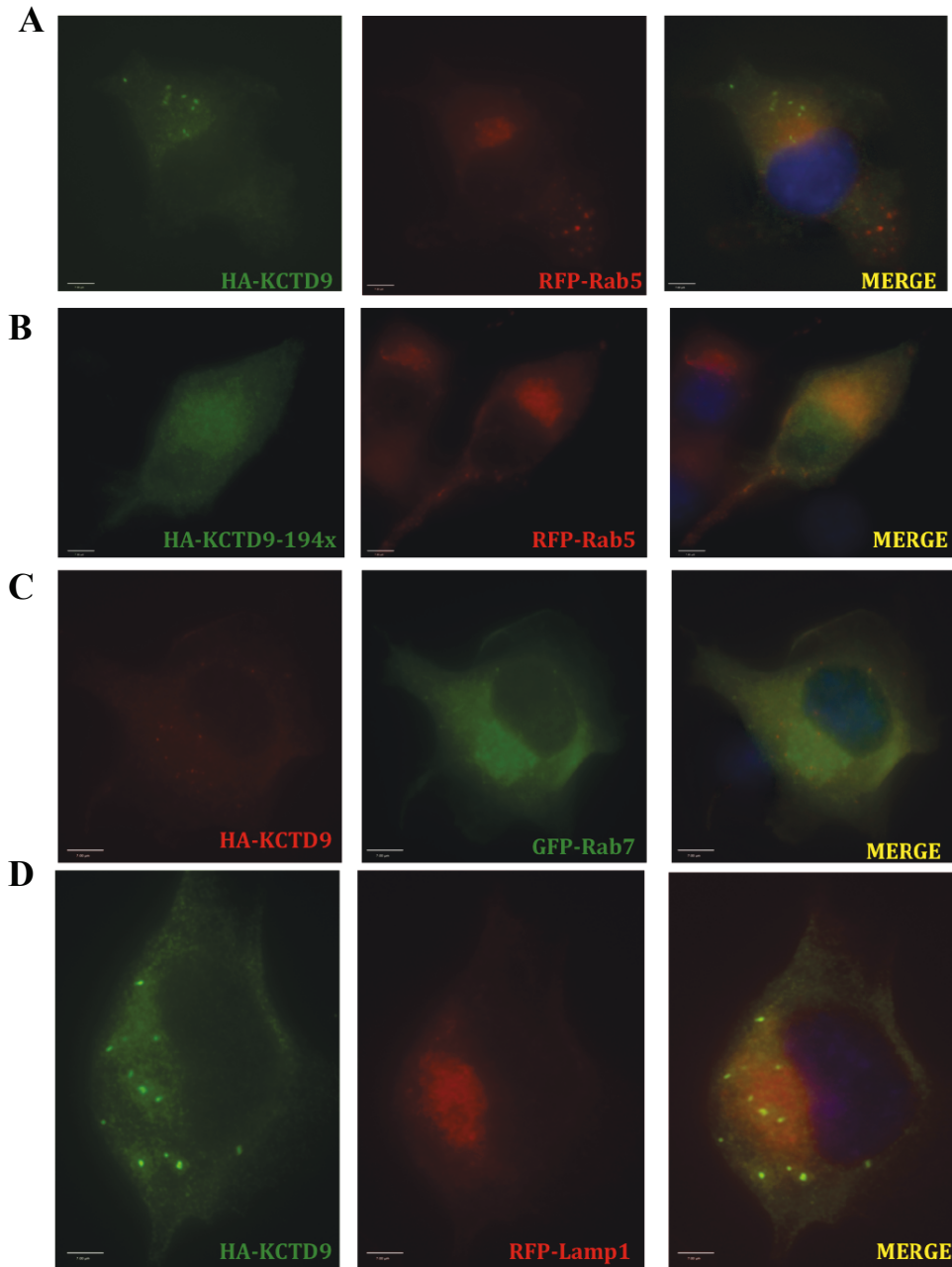


Figure 6. KCTD9 does not co-localize with endosomal markers. (A) HA-KCTD9 (anti-HA, green) co-expressed with RFP-Rab5 (direct fluorescence, red); nuclei stained with DAPI (blue). (B) HA-KCTD9-194x (anti-HA, green) co-expressed with RFP-Rab5 (direct fluorescence, red); nuclei stained with DAPI (blue). (C) HA-KCTD9 (anti-HA, red) co-expressed with GFP-Rab7 (anti-GFP, green); nuclei stained with DAPI (blue). (D) HA-KCTD9 (anti-HA, green) co-expressed with RFP-Lamp1 (direct fluorescence, red); nuclei stained with DAPI (blue).

Autophagy Induction Markers		
Condition	No treatment	Treatments (Rapamycin or Starvation Media)
mTOR-myc (negative regulator)	No co-localization	No co-localization
ULK1-myc	No co-localization	No co-localization
Atg13-myc	No co-localization	No co-localization
Vesicle Nucleation Markers		
Beclin1-dsRed	Partial co-localization	-
Vps34-FLAG	Co-localization	Co-localization
GFP-Rubicon (negative regulator)	Partial co-localization	-
FYVE-dsRed	No co-localization	No co-localization
WIPI-1	No co-localization	-
Expansion Markers		
mCherry-Atg5	Partial co-localization	Partial co-localization
GFP-Atg12	Partial co-localization	Partial co-localization
GFP-GATE16	No co-localization	No co-localization
RFP-LC3	Partial co-localization	Co-localization

Table 2. Summary of co-localization results between autophagy markers and KCTD9. Numerous immunofluorescence experiments were performed in order to determine autophagy markers that KCTD9 co-localizes with as outlined above. These experiments were performed in COS7 and N2a cells with no treatment or an autophagy stimulus.

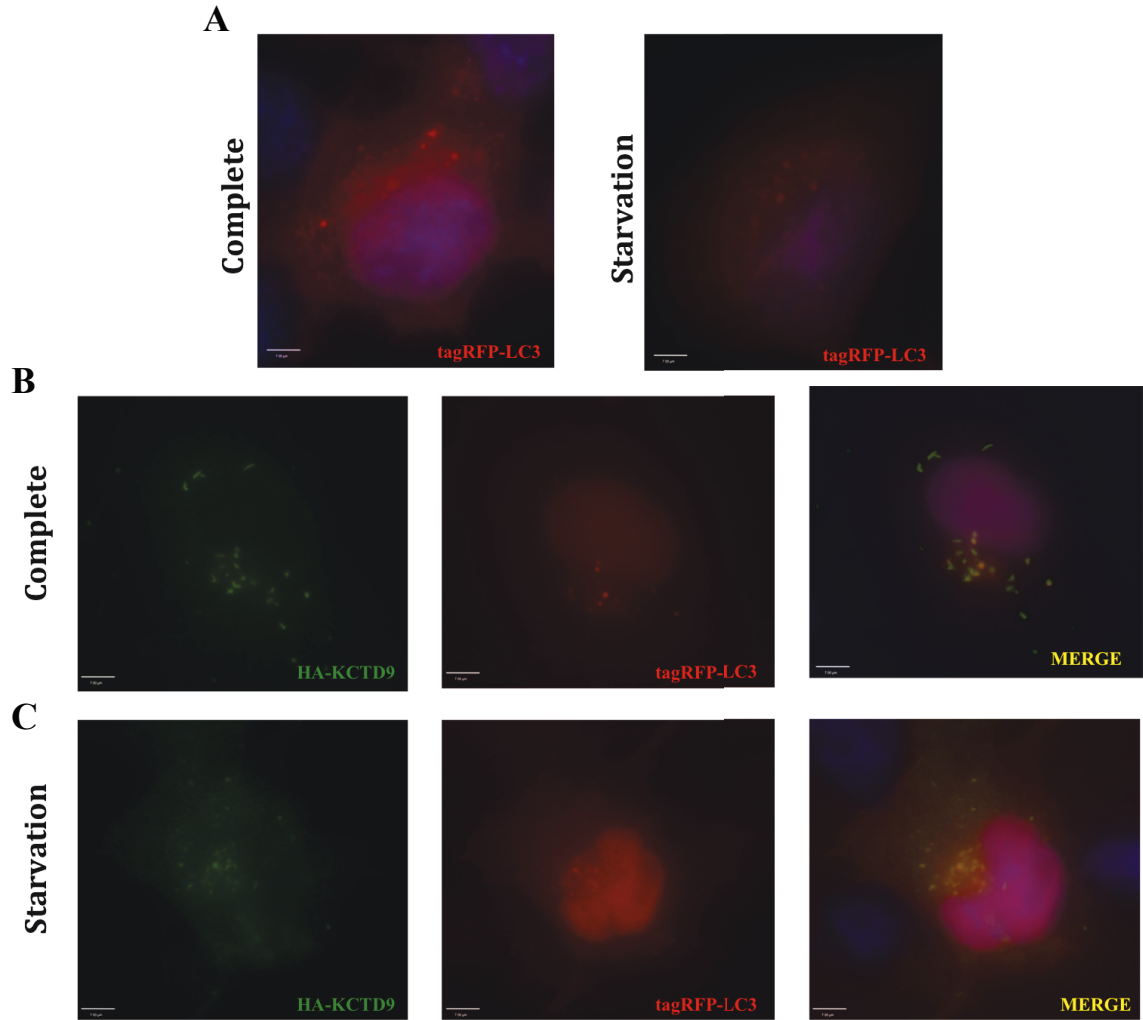


Figure 7. KCTD9 co-localizes with autophagosome marker LC3. (A) Immunofluorescence microscopy of COS7 cells transfected with tagRFP-LC3 (direct fluorescence, red) in complete or amino acid and serum free media for 5 h; nuclei stained with DAPI (blue). (B) Co-expression of HA-KCTD9 (anti-HA, green) and tagRFP-LC3 (direct fluorescence, red) 19h post-transfection; nuclei stained with DAPI (blue) (C) Co-expression of HA-KCTD9 (anti-HA, green) and tagRFP-LC3 (direct fluorescence, red) 19h post-transfection after 5 h starvation; nuclei stained with DAPI (blue).

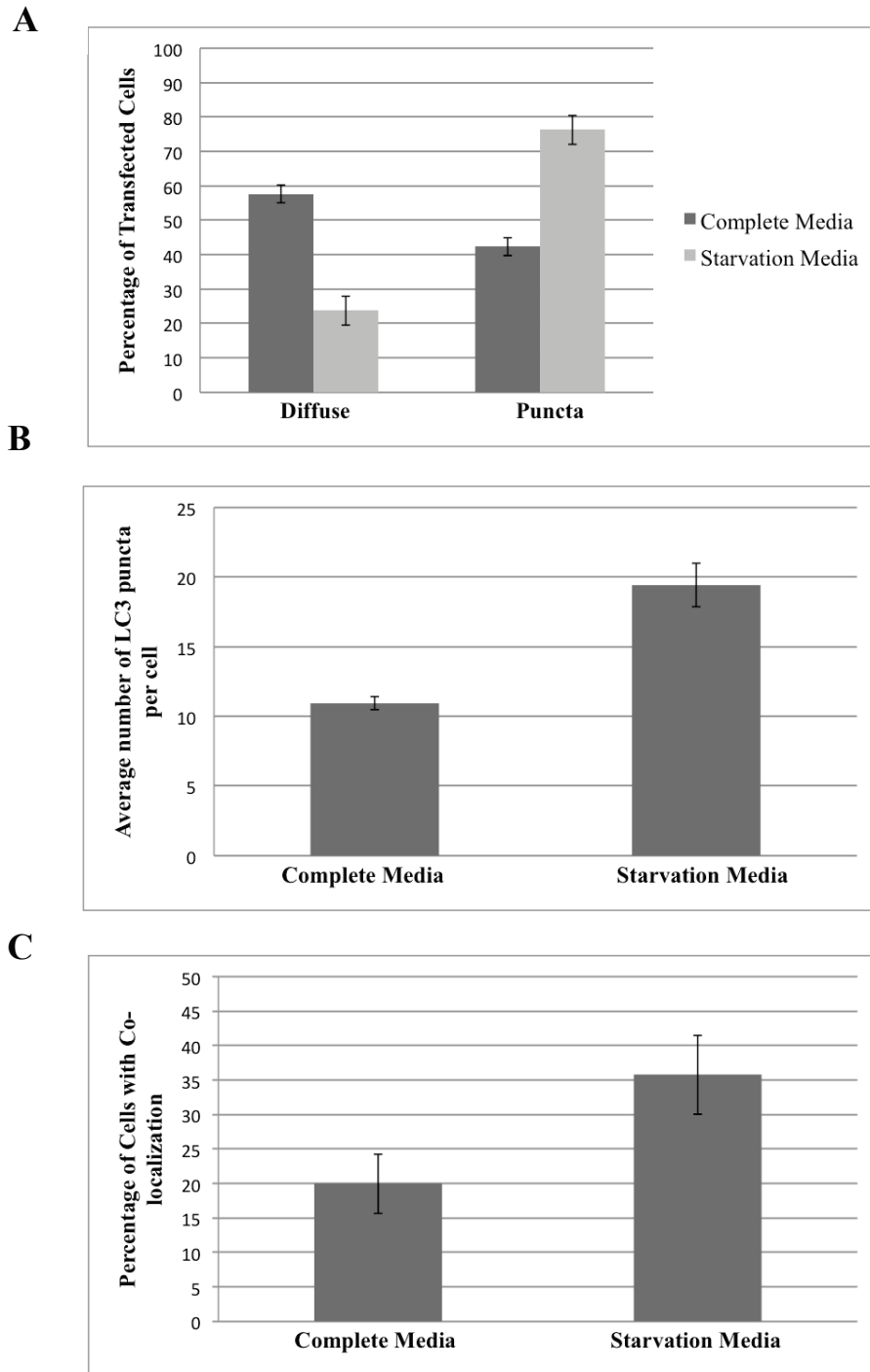


Figure 8. Starvation induces LC3 puncta and increases co-localization with KCTD9. (A) Quantification of COS7 cells transfected with tagRFP-LC3 comparing diffuse and puncta in complete and starvation media. (B) Number of LC3 puncta averaged in 25 cells per experiment (n=3). (C) Quantification of co-localization between tagRFP-LC3 and HA-KCTD9 in COS7 cells (n=3).

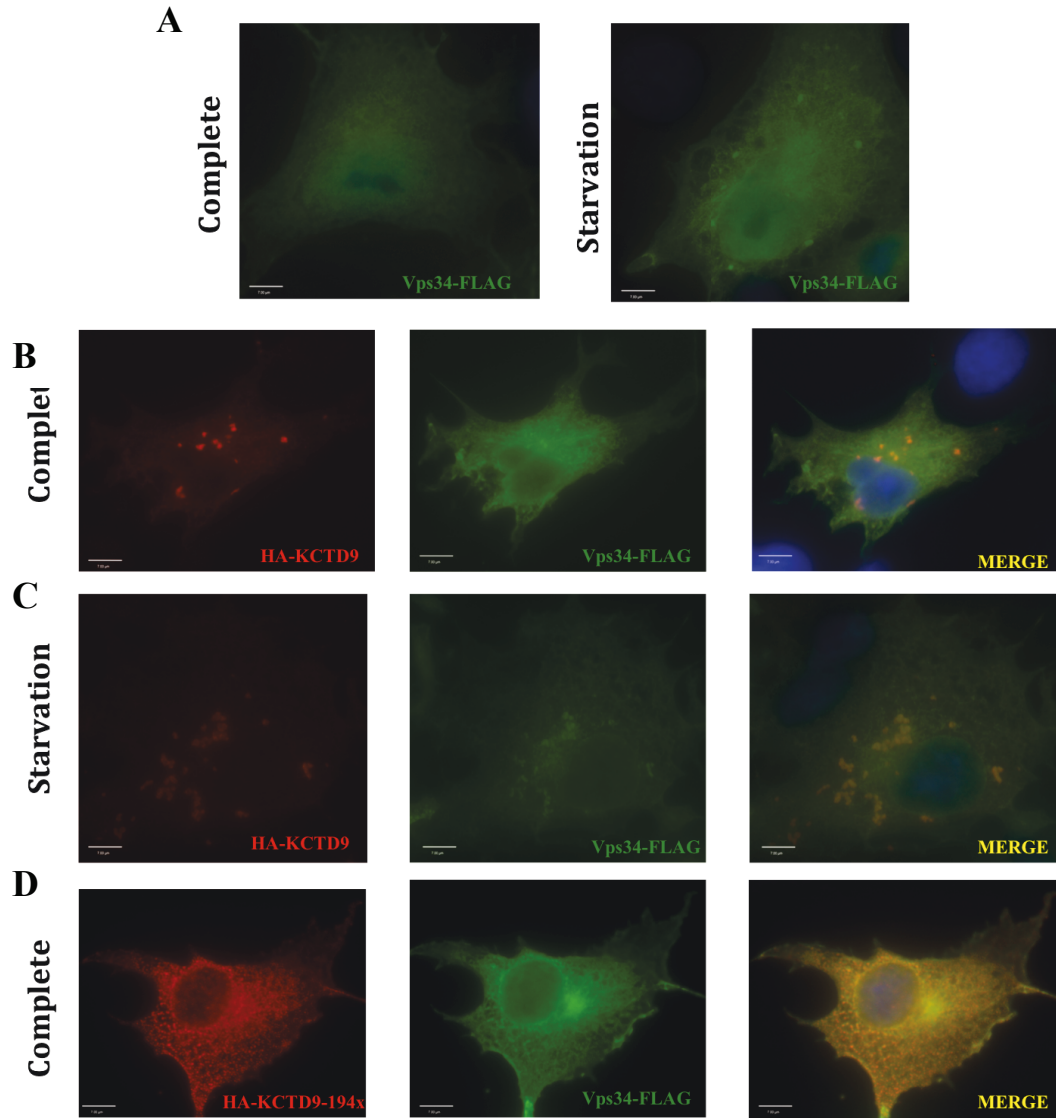


Figure 9. KCTD9 co-localizes with early autophagosome marker Vps34. (A) Immunofluorescence microscopy of COS7 cells transfected with Vps34-FLAG (anti-FLAG, green) in complete or amino acid and serum free media for 5 h; nuclei stained with DAPI (blue). (B) Co-expression of HA-KCTD9 (anti-HA, red) and Vps34-FLAG (anti-FLAG, green) 19h post-transfection; nuclei stained with DAPI (blue) (C) Co-expression of HA-KCTD9 (anti-HA, red) and Vps34-FLAG (anti-FLAG, green) 19h post-transfection after 5 h starvation; nuclei stained with DAPI (blue).

A

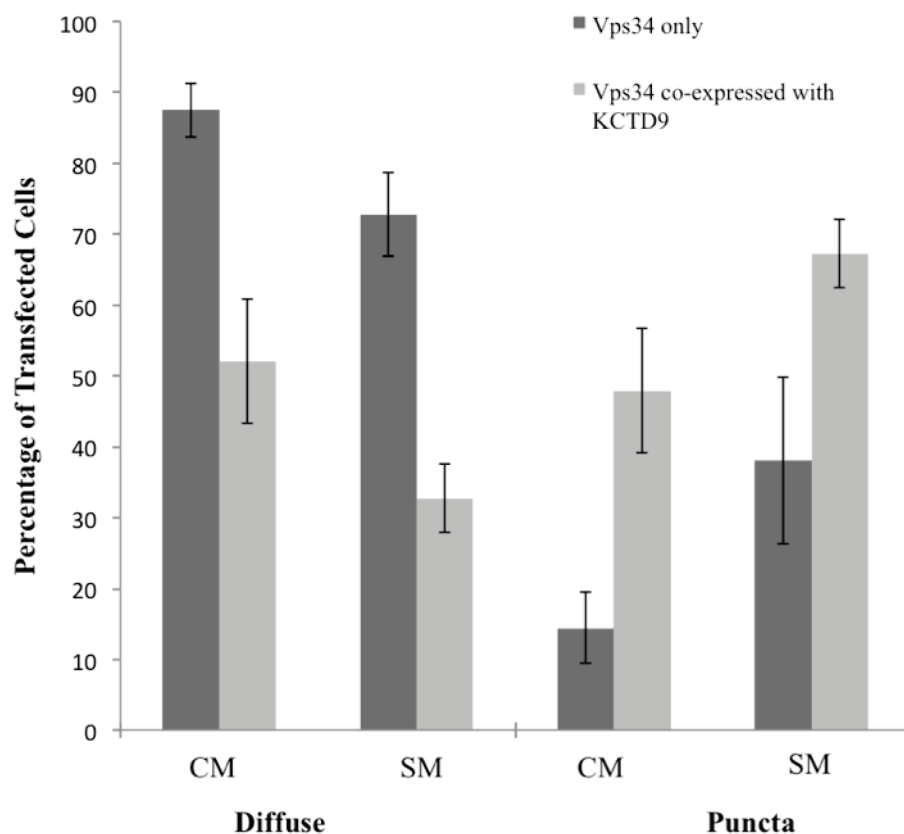


Figure 10. KCTD9 induces Vps34 puncta in complete and starvation media. (A) Quantification of immunofluorescence microscopy of COS7 cells co-transfected with Vps34-FLAG and HA-KCTD9 in complete media or media to stimulate autophagy. CM: complete media. SM: media with 50 nM rapamycin for 2 hours or amino acid and serum free media for 4 hours. (n=3).

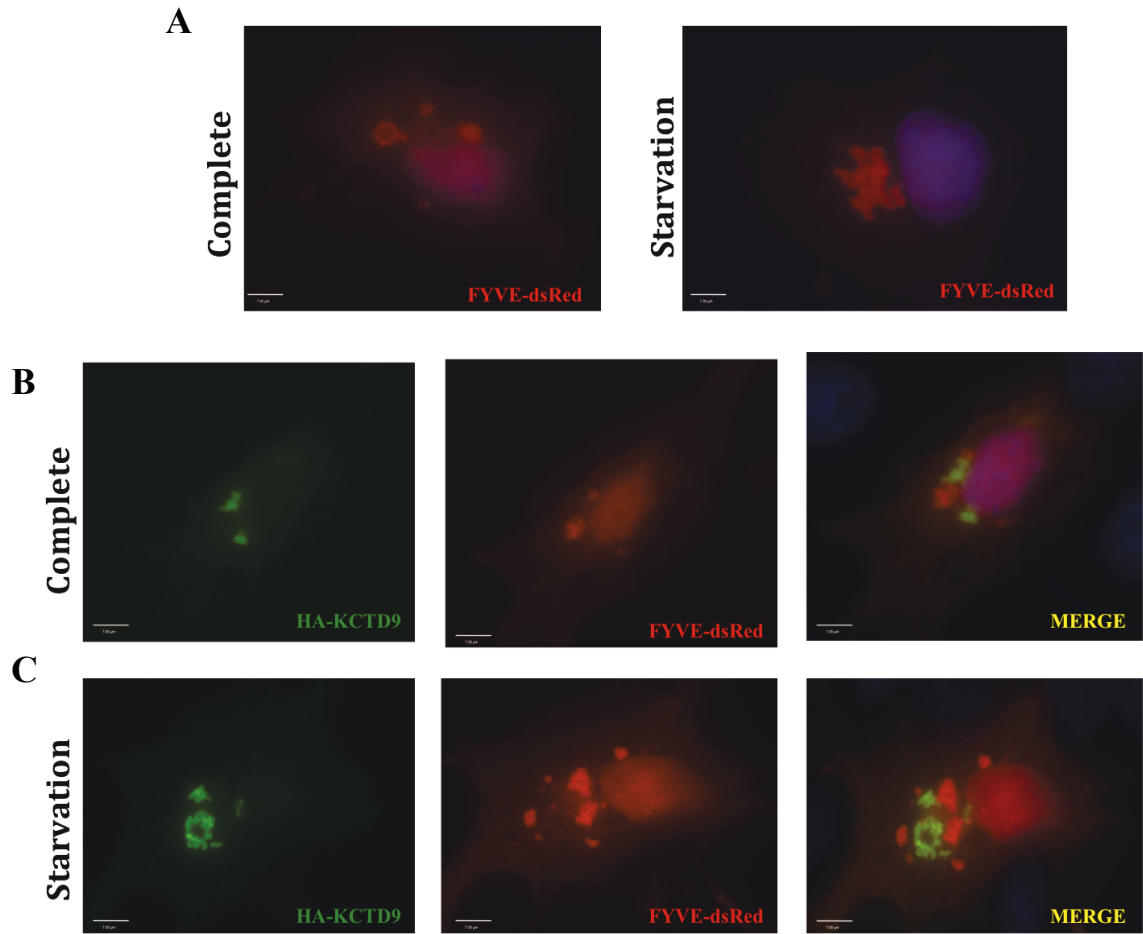


Figure 11. KCTD9 does not co-localize with FYVE-domain. (A) Immunofluorescence microscopy of COS7 cells transfected with FYVE-dsRed (direct fluorescence, red) in complete or amino acid and serum free media for 5 h; nuclei stained with DAPI (blue). (B) Co-expression of HA-KCTD9 (anti-HA, green) and FYVE-dsRed (direct fluorescence, red) 19h post-transfection; nuclei stained with DAPI (blue) (C) Co-expression of HA-KCTD9 (anti-HA, green) and FYVE-dsRed (direct fluorescence, red) 19h post-transfection after 5 h starvation; nuclei stained with DAPI (blue).

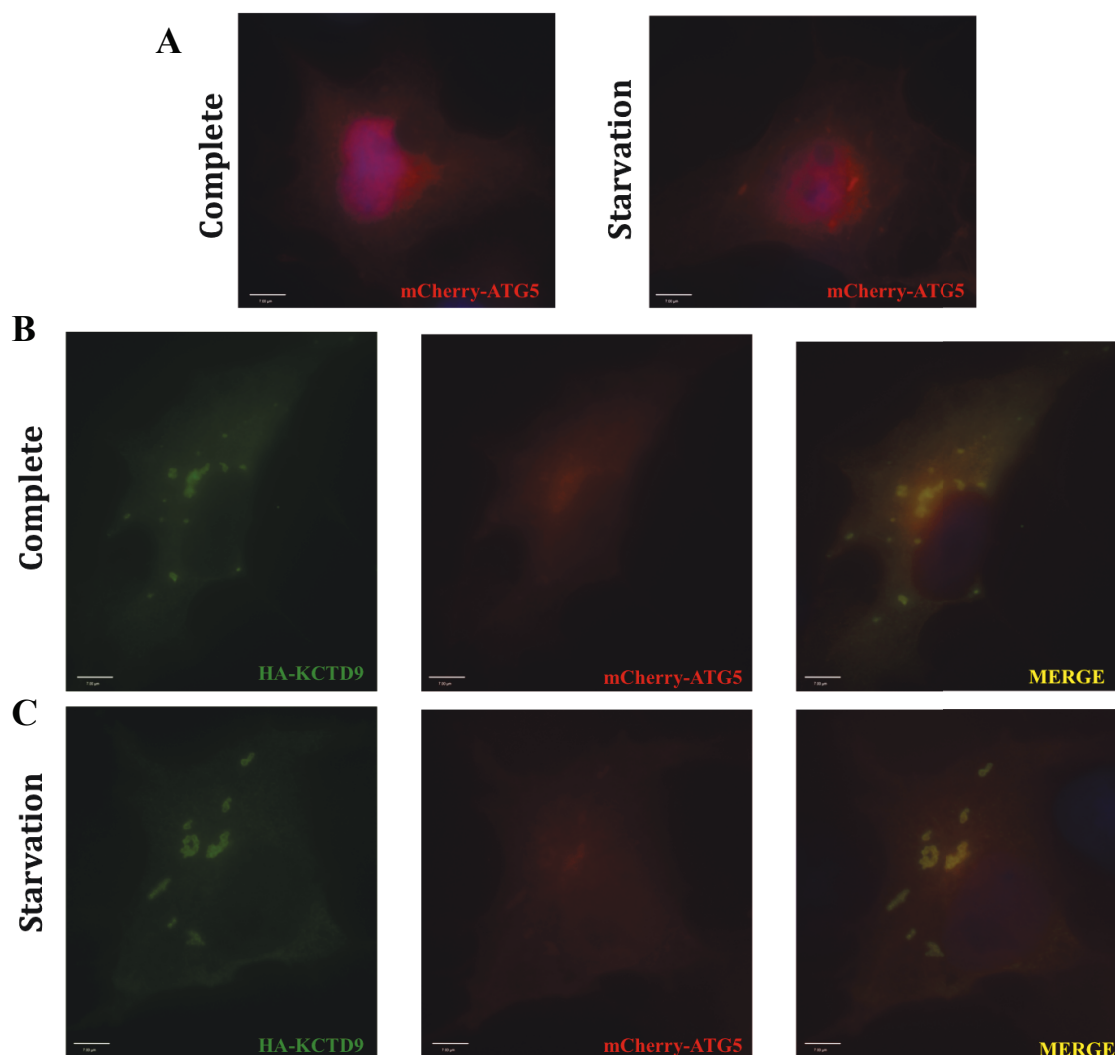


Figure 12. KCTD9 co-localizes with autophagy protein Atg5. (A) Immunofluorescence microscopy of COS7 cells transfected with mCherry-ATG5 (direct fluorescence, red) in complete or amino acid and serum free media for 5 h; nuclei stained with DAPI (blue). (B) Co-expression of HA-KCTD9 (anti-HA, green) and mCherry-ATG5 (direct fluorescence, red) 19h post-transfection; nuclei stained with DAPI (blue) (C) Co-expression of HA-KCTD9 (anti-HA, green) and mCherry-ATG5 (direct fluorescence, red) 19h post-transfection after 5 h starvation; nuclei stained with DAPI (blue).

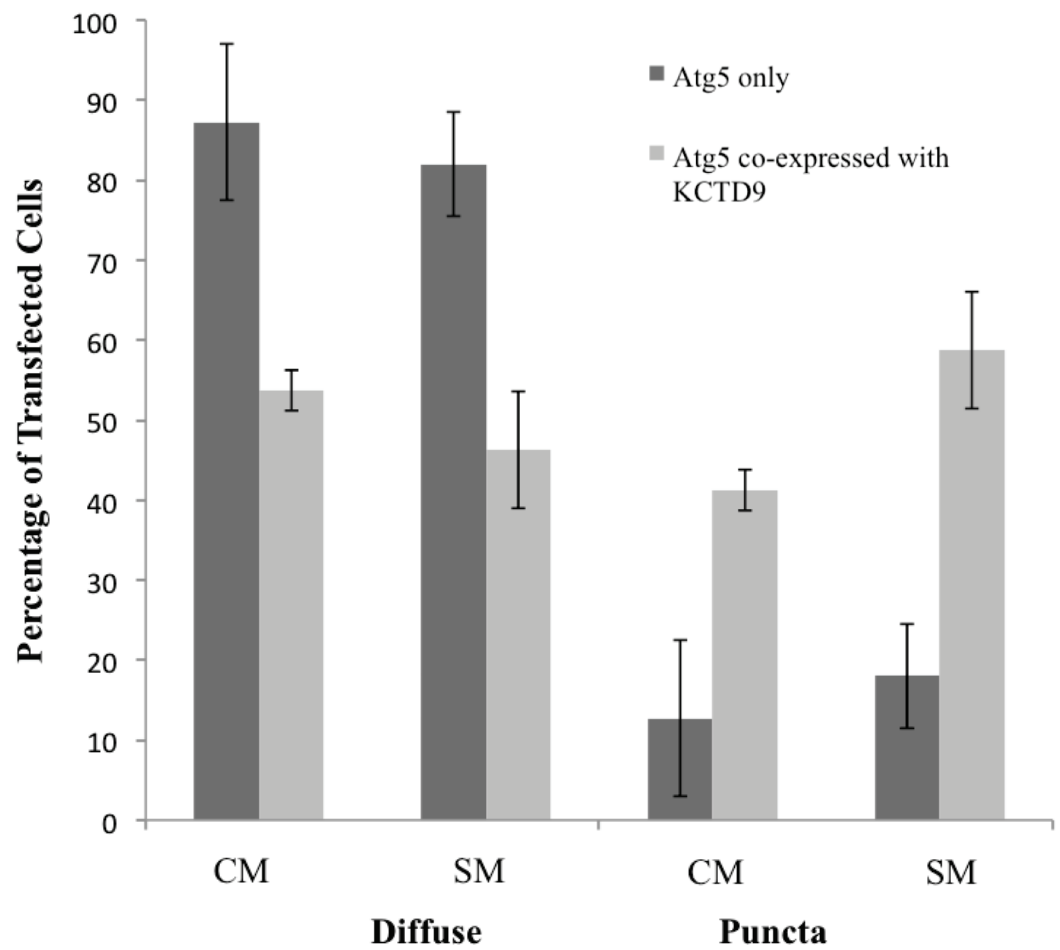
A

Figure 13. KCTD9 induces Atg5 puncta in complete and starvation media. (A) Quantification of immunofluorescence microscopy of COS7 cells co-transfected with mCherry-Atg5 and HA-KCTD9 in complete media or media to stimulate autophagy. CM: complete media. SM: media with 50 nM rapamycin for 2 hours or amino acid and serum free media for 4 hours. (n=3).

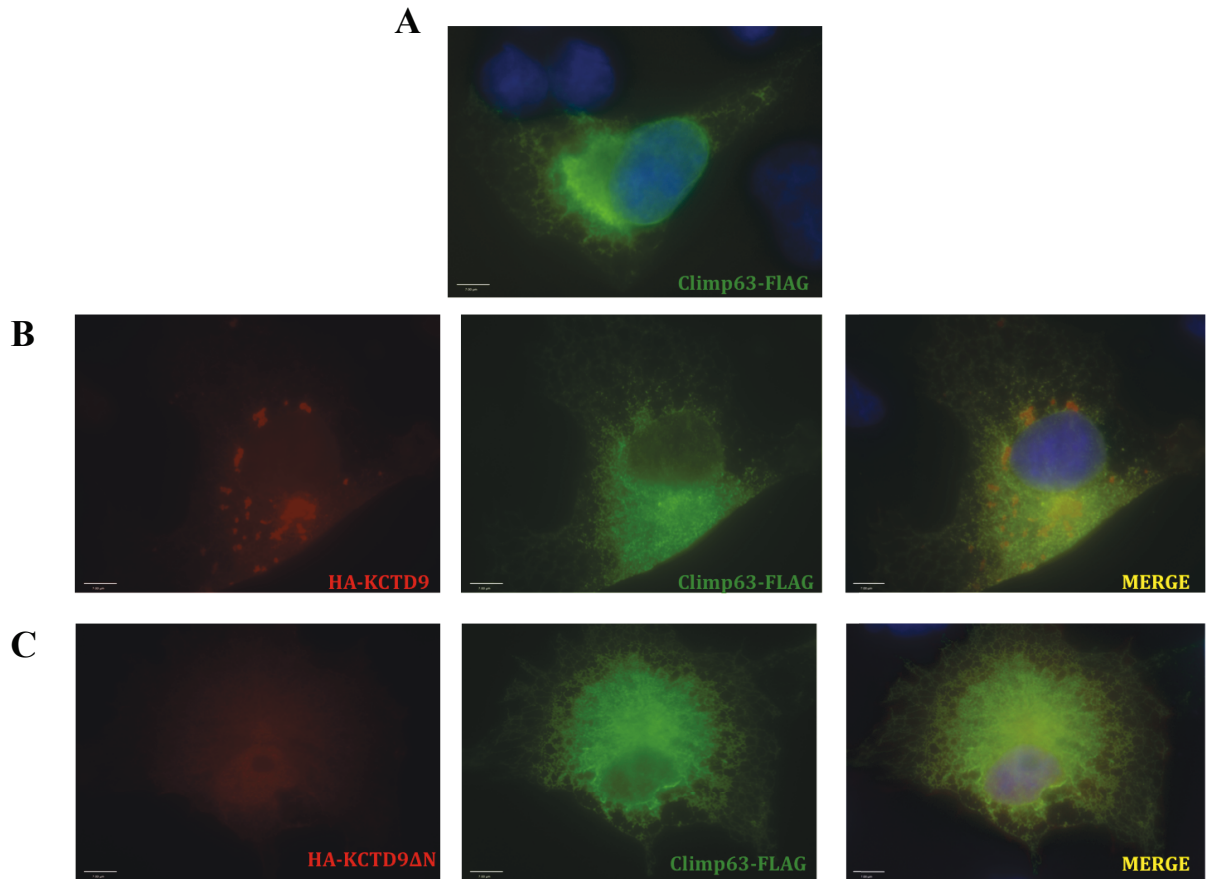


Figure 14. KCTD9 full-length and mutant construct associate with endoplasmic reticulum marker Climp63. (A) Immunofluorescence microscopy of COS7 cells transfected with Climp63-FLAG (anti-FLAG, green); nuclei stained with DAPI (blue). (B) Co-expression of HA-KCTD9 (anti-HA, red) and Climp63-FLAG (anti-FLAG, green) post 19 h; nuclei stained with DAPI (blue) (C) Co-expression of HA-KCTD9ΔN (anti-HA, red) and Climp63-FLAG (anti-FLAG, green); nuclei stained with DAPI (blue).

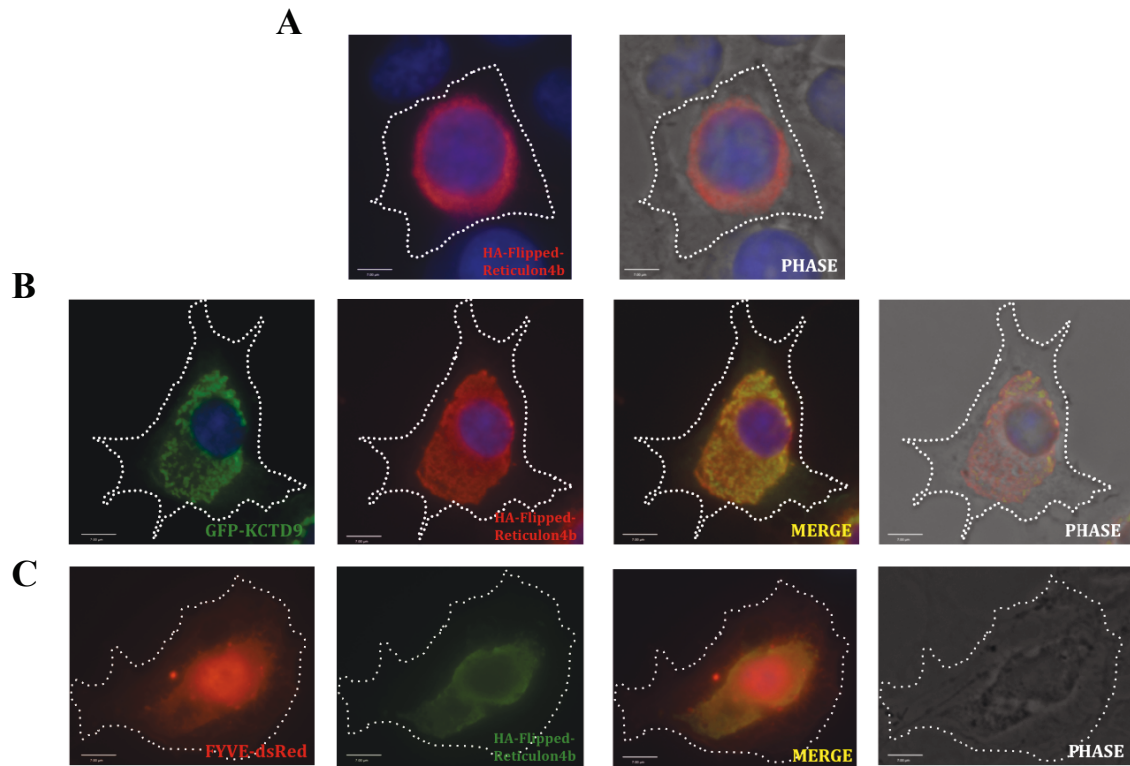


Figure 15. KCTD9 localizes to subdomains of the endoplasmic reticulum in COS7 cells. (A) Immunofluorescence microscopy of COS7 cells transfected with Flipped-Reticulon4b (anti-HA, red); detected by DAPI (blue), confocal phase image presented. (B) GFP-KCTD9 (anti-GFP, green) co-expressed with HA-Flipped-Reticulon4b (anti-HA, red); nuclei detected by DAPI (blue), confocal phase image presented. (C) FYVE-dsRED (direct fluorescence, red) co-expressed with HA-Flipped-Reticulon4b (anti-HA, green); nuclei detected by DAPI (blue), confocal phase image presented.

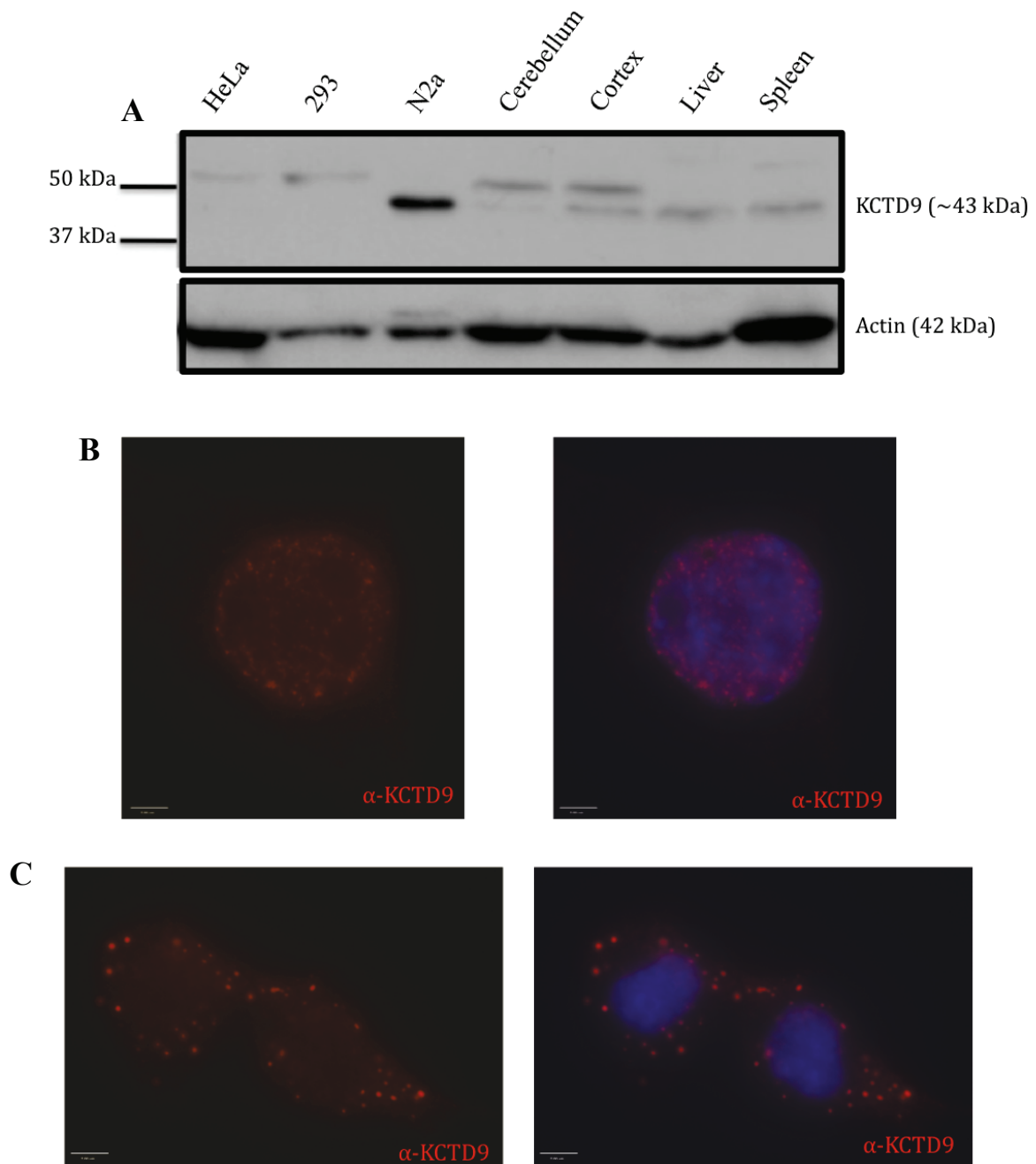


Figure 16. Endogenous expression of KCTD9 in N2a cells. (A) Use of GeneTex anti-KCTD9 antibody for endogenous detection - high level of expression in N2a cells. (B) Endogenous detection of KCTD9 in N2a cells - puncta in nucleus. (C) Endogenous detection of KCTD9 in N2a cells - puncta in the cytoplasm.

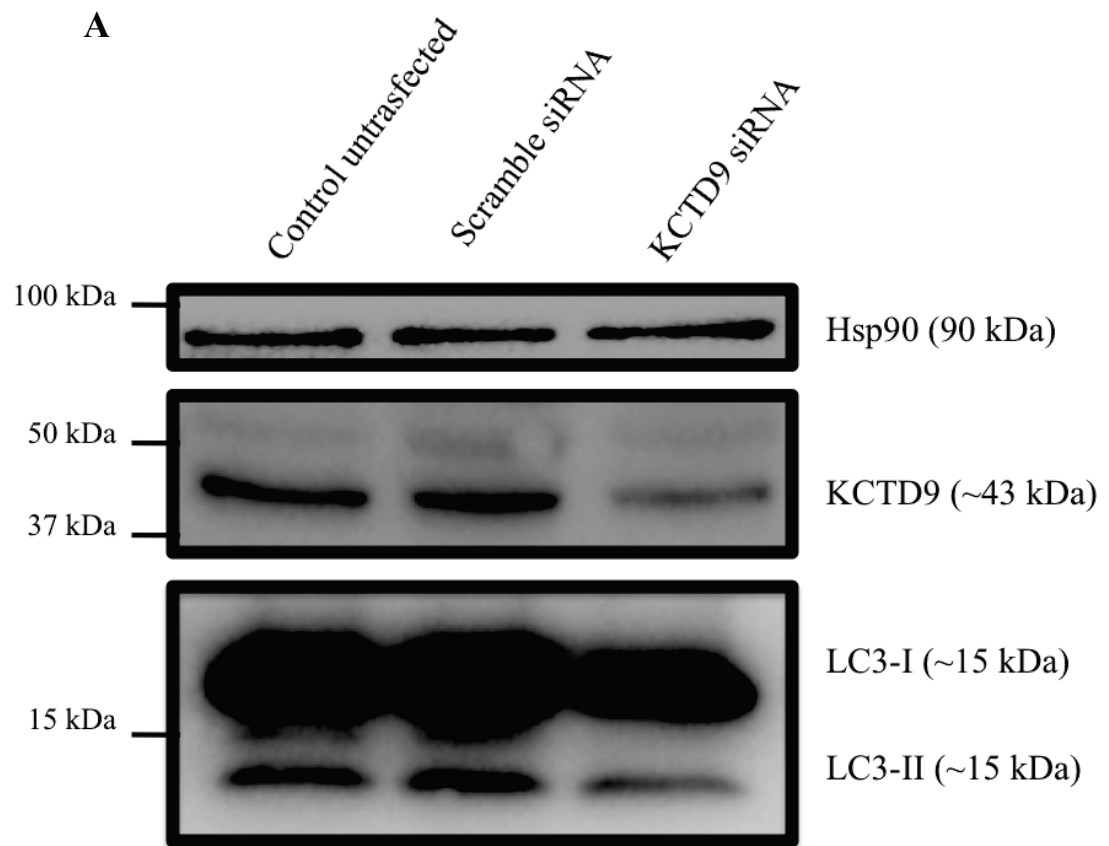


Figure 17. KCTD9 siRNA knockdown leads to possible autophagy induction defect. (A) Dharmacon siRNA smartpool for KCTD9 was used in order to knockdown KCTD9 in N2a cells. While there was not total knockdown, there was a change in LC3-I and LC3-II levels when KCTD9 was partially knocked down indicating a possible defect between the conversion of LC3-I to LC3-II.

A

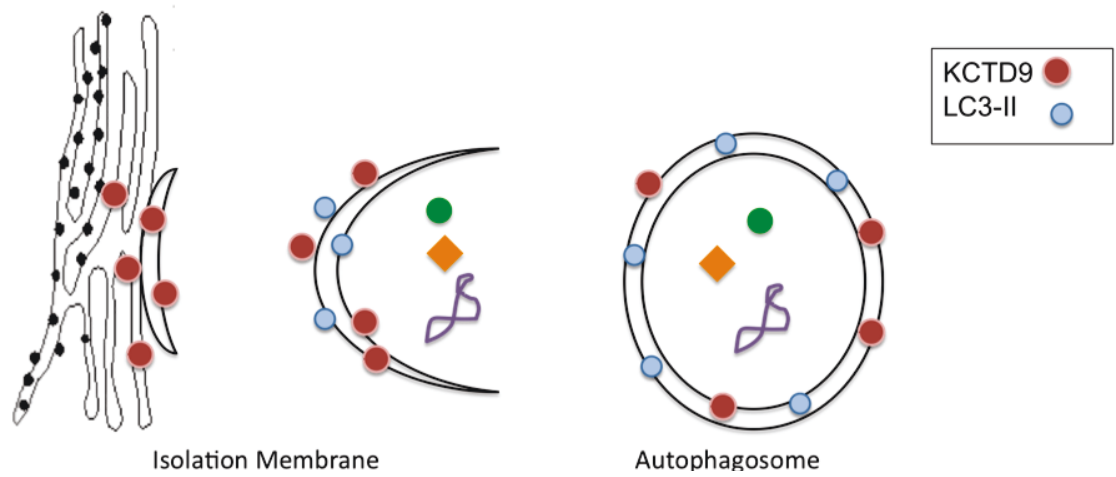


Figure 18. Model of KCTD9's potential role in the autophagy pathway. (A) KCTD9 localizes to certain subdomains of the ER where the autophagosomes develops from. KCTD9 may be playing a role in inducing pre-autophagosomal sites and promoting the development of autophagosomes. Therefore co-localization is seen with Vps34, Atg5, and LC3. Furthermore, knocking down KCTD9 leads to a defect in formation of autophagosomes suggesting a role of KCTD9 in early autophagosome synthesis.

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Education

Expected 2014

Master of Science (MSc), Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Thesis: The role of human KCTD9 in the autophagy pathway

05/2012

Bachelor of Science (BSc), Anatomy and Cell Biology, McGill University, Montreal, QC

06/2008

High School Graduation Diploma, University of Toronto Schools, Toronto, ON
Lieutenant Governor's Community Volunteer Award

Professional Training

05/2014

Global Health Certificate, Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Technical Training

02/2014

Johns Hopkins University, Baltimore, Maryland

- EPR View – 01
- Basic Human Subjects Research

01/2011

McGill University, Montreal, QC

- W.H.M.I.S. Training
- Basic Course on Animal Use for Research and Training
- Mouse Methodology Workshop - Module 1

Research Experience

11/2012 - Present

Master's Thesis Project, Hardwick Lab, Baltimore, MD

- Determining the biological function of understudied human KCTD proteins that are associated with cancer and neurodegenerative diseases
- Testing the hypothesis of KCTD proteins involved in regulating the autophagy pathway

- Understanding the function of the KCTD proteins will allow for the development of suitable therapeutic strategies

09/2010 - 04/2012

Research Assistant, Komarova Lab, Montreal, QC

- Studying osteoclast expression to better understand diseases such as osteoporosis and rheumatoid arthritis
- Investigating the effects of Jak3 and Jak2/Stat3 Jak2/Stat3 inhibitors on osteoclast formation
- Determining the Epo receptor signaling pathway to better control osteoclast proliferation

Presentations

Ladha F, Teng, X, Hardwick, JM. Potential role of human KCTD9 in the autophagy pathway. Presented at Delta Omega Poster Presentation, Baltimore, MD, 2014.

Leadership Experience

06/2013 - Present

09/2012 - 05/2013

VP Communications

Member-at-Large

Student Assembly: Bloomberg School of Public Health, Baltimore, MD

- Elected position responsible for serving on Social and Cultural Affairs and Student Groups Committees
- Organizing social events, reviewing student group applications, and the Dean's dinner

09/2011 - 05/2012

09/2010 - 05/2011

President

Exam-AID Coordinator

Students Offering Support: McGill Chapter, Montreal, QC

- Organizing first and second year tutorial services to raise money for developmental projects in Latin America
- Responsible for selecting qualified executive members, tutors while overseeing club finances, marketing, and sponsorship

09/2011 - 05/2012

09/2010 - 05/2011

President

VP Communications

Fine Arts Club, Montreal, QC

- Promoting visual arts in the McGill community by organizing events including painting workshops and art gallery visits
- Regularly communicating with executive members and keeping club members informed of events

08/2008 - 04/2009

Sponsorship Committee Executive

Science Undergraduate Society (SUS), Montreal, QC

- Assisted CEO in establishing business ties and obtaining sponsorship for SUS events
- Contacted various companies for the organization and implementation of a Career and Networking Fair

Volunteer Experience

08/2013 - Present

High School Mentor

Incentive Mentoring Program, Baltimore, MD

- Providing academic and social support to under performing youths who are struggling with poverty, drugs, and violence
- Instilling self-confidence and motivation through homework and college application assistance

01/2010 - 04/2012

Biology Tutor

Students Offering Support McGill, Montreal, Quebec

- Compiling relevant course and clearly communicating major concepts during review sessions to thirty to forty students
- Donating all money raised towards developmental projects in Latin America

02/2009 - 04/2010

Student Volunteer

Mount Sinai Hospital, Montreal, Quebec

- Assisted patients during meal times and made sure each patient received appropriate meal and snack
- Spent time with patients during visiting hours and accompanied them to activities such as jeopardy and bingo

05/2009 - 08/2009

Children's Department Volunteer

Dr. Roz's Healing Place, Toronto, Ontario

- Worked with abused children and helped with routine activities and homework
- Accompanied children during field trips and supported mothers during meal time and childcare workshops